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The role of insulin-like growth factors in the fetus.

Mark Hope Oliver.

A thesis in partial fulfilment of the requirements for the degree of Doctor of Philosophy,
University of Auckland.

1994
ABSTRACT

Fetal substrate supply is important in the regulation of fetal growth. The insulin-like growth factors (IGFs), insulin and placental lactogen (PL) may be important mediators in fetal growth regulation. However, nutritional regulation of circulating concentrations of IGFs, their binding proteins (IGFBPs) and PL during fetal life is poorly understood. The objective of this thesis is to characterise the influence of nutrition on the regulation of these hormones in the fetal circulation.

Pregnant ewes were starved for 72 h and then refed for 48 h. After 48 h starvation fetuses were infused with glucose, amino acids or insulin while maternal starvation continued. Other fetuses of fed mothers were infused with recombinant oPL or IGF-I for 24 h.

Fetal blood glucose, plasma IGF-I, IGF-II and insulin concentrations all fell on maternal starvation. Fetal plasma IGF-I and insulin concentrations returned to near control values on fetal infusion of glucose or insulin but not amino acids suggesting glucose had a more important role than amino acids in plasma IGF-I regulation and that insulin may have mediated the effect of glucose. Fetal plasma IGF-II concentrations returned to control values on fetal infusion of glucose, but not insulin. Thus fetal plasma IGF-I and IGF-II concentrations appear to be regulated differently by fetal glucose and insulin.

Fetal plasma IGFBP-1 and BP-2 levels increased, while fetal plasma IGFBP-3 and type 2 receptor levels decreased, on maternal starvation. Fetal infusion of glucose or insulin returned fetal plasma IGFBP-1 to near control levels. All fetal plasma IGFBPs were near control levels after maternal refeeding. Fetal plasma IGFBPs and type 2 IGF receptor levels were influenced by nutrition but IGFBP-2, BP-3 and the circulating type 2 IGF receptor appear to be at least partly regulated by factors other than glucose and insulin. In a separate experiment fetal plasma IGF-I concentrations increased on IGF-I infusion to fetuses whose mothers were on a low plane of nutrition but did not increase in fetuses whose mothers were on a high plane of nutrition suggesting that increases in fetal plasma IGFBP concentrations on maternal starvation or undernutrition may increase fetal plasma IGF-carrying capacity.
Plasma concentrations of PL in fetuses whose mothers had been on a high plane of nutrition was higher during starvation than in fetuses whose mothers had been on a low plane of nutrition. Fetal plasma PL concentrations increased 3-fold on maternal infusion of glucose at the end of starvation but was unaffected by fetal infusion of glucose at a lower rate. Fetal plasma PL responses to starvation were influenced by prior nutritional status. The rate of glucose infusion appears to determine the occurrence of a fetal plasma PL response. Fetal blood amino acid nitrogen (AN) concentrations fell on fetal infusion of oPL but fetal blood glucose, plasma IGF-I, IGF-II and insulin concentrations were unaffected. PL does not appear to have a role in the endocrine regulation of the IGFs in fetal sheep but may influence fetal amino acid metabolism.

In these studies the fetal plasma concentrations of the IGFs, insulin, PL and plasma levels of IGFBP-1 to -4 have been demonstrated to be regulated by fetal substrate supply. These experiments provide further evidence that these hormones may be mediators in the regulation of fetal growth by fetal substrate supply.
ACKNOWLEDGMENTS.

I would firstly like to thank Dr Jane Harding and Prof. Peter Gluckman for their encouragement and supervision in these studies. Dr Bernhard Breier and Mr Brian Gallaher have been helpful in the analysis of endocrine samples. I would like to acknowledge Mrs Pandora Evans for her effort in developing and supervising assays for the analysis of blood metabolites. Dr Wieland Kiess of the Children's Hospital, University of Munich kindly offered to analyse plasma samples for the circulating type 2 receptor. I would like to thank Christine Gibson, Fiona Ffolliott-Powell, Dr Li Liu, Mark Vickers and other staff of the Research Centre for Developmental Medicine and Biology for their technical assistance and help. None of these studies would have been possible without the help of the dedicated staff of the Animal Resources Unit of Auckland School of Medicine and the Reproductive Biology Unit of Ruakura Animal Research Centre. I am grateful to The Foundation for the Newborn (NZ) for their support throughout the course of my studies.
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<td>ALP</td>
<td>Acid labile protein</td>
</tr>
<tr>
<td>AN</td>
<td>Amino acid nitrogen</td>
</tr>
<tr>
<td>bGH</td>
<td>Bovine growth hormone</td>
</tr>
<tr>
<td>BP</td>
<td>Binding protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Coeff. Var.</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CRL</td>
<td>Crown rump length</td>
</tr>
<tr>
<td>d</td>
<td>Day(s)</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>des</td>
<td>Desipeptide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxribonucleic acid</td>
</tr>
<tr>
<td>ECBP</td>
<td>Endothelial cell insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>hGH</td>
<td>Human growth hormone</td>
</tr>
<tr>
<td>hPL</td>
<td>Human placental lactogen</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin-like growth factor-I</td>
</tr>
<tr>
<td>IGF-II</td>
<td>Insulin-like growth factor-II</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>M</td>
<td>Moles per litre</td>
</tr>
<tr>
<td>mg</td>
<td>Milligrams</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitres</td>
</tr>
<tr>
<td>mM</td>
<td>Millimoles per litre</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>n</td>
<td>Number</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>ng/ml</td>
<td>Nanogram per millilitre</td>
</tr>
<tr>
<td>ng/tube</td>
<td>Nanogram per tube</td>
</tr>
<tr>
<td>oCS</td>
<td>Ovine chorionic somatommmamotropin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>ODC</td>
<td>Ornithine decarboxylase</td>
</tr>
<tr>
<td>oGH</td>
<td>Ovine growth hormone</td>
</tr>
<tr>
<td>oPL</td>
<td>Ovine placental lactogen</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pCO₂</td>
<td>Partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PL</td>
<td>Placental lactogen</td>
</tr>
<tr>
<td>pO₂</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PP12</td>
<td>Placental protein 12</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
</tr>
<tr>
<td>rGH</td>
<td>Rat growth hormone</td>
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<tr>
<td>rh-insulin</td>
<td>Recombinant human insulin</td>
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<tr>
<td>rhIGF-I</td>
<td>Recombinant human insulin-like growth factor -I</td>
</tr>
<tr>
<td>rhIGF-II</td>
<td>Recombinant human insulin-like growth factor -II</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>rPL</td>
<td>Rat placental lactogen</td>
</tr>
<tr>
<td>rPRL</td>
<td>Rat prolactin</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>μCi/μg</td>
<td>Microcuries per microgram (specific activity)</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre</td>
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CHAPTER 1. INTRODUCTION.

SIGNIFICANCE OF FETAL GROWTH.

Intrauterine growth retardation is a leading cause of perinatal mortality and morbidity. Infants that are small at birth and then do not exhibit catch up growth are at high risk of long-term physical and neurological morbidity (Fancourt, Campbell, Harvey & Norman 1976). Impaired fetal growth appears to have strong association with the development of hypertension and diabetes later in adult life (Barker 1992). Fetal substrate availability is the most important factor in the regulation of fetal growth (Owens 1991; Gluckman & Harding 1992). Insulin-like growth factor (IGF)-I, IGF-II, placental lactogen (PL) and insulin are also suggested to have important roles in the regulation of fetal growth (Gluckman, Breier, Oliver, Harding & Bassett 1990; Owens 1991; Gluckman & Harding 1992). The aim of this thesis is to characterise the nutritional regulation of these hormones in the fetal circulation.

FETAL GROWTH & NUTRITION.

In the sheep fetus, fetal growth continues steadily until about 120 d gestation (term = 147 d) when the growth rate tends to slow (Mellor 1983). Fetal growth, like postnatal growth, can be limited by genetic factors but in late gestation fetal substrate supply is the main regulator of growth. Severe undernutrition of the pregnant ewe in late gestation resulted in a prompt decrease in the rate of fetal growth as measured by indwelling crown rump and girth devices (Mellor & Murray 1982). Refeeding of the ewe up to 16 d from the onset of undernutrition resulted in a prompt return to the normal rate of growth. Similarly, starvation of pregnant rats in late gestation leads to fetal growth retardation (Straus, Ooi, Orlowski & Rechler 1991).

The ability of the mother and her uterus to maintain fetal substrate supply is a normal constraining factor on fetal growth in late gestation and this is probably why growth of the fetal lamb slows in the last 20-30 d of gestation. Maternal constraint is illustrated in the relationship between fetal number and fetal weight (Kroonsberg, McCutcheon, Siddiqui, Mackenzie, Blair, Ormsby, Breier & Gluckman 1989). In experiments where an
embryo from a large strain of a breed is transferred into the uterus of smaller strain and allowed to reach term the birthweight is much lower than would be expected if the embryo had continued to develop in the uterus of its own strain (Snow 1989). These experiments demonstrate that the uterine environment appears to be an important constraining factor on growth in late gestation despite the genetic potential of the fetus.

Placental growth in the sheep peaks at around 80-90 d gestation and as this organ regulates the usage and supply of substrates taken up from the uterine circulation its size may be an important determinant of fetal growth. Surgical reduction in the number of placental implantation sites prior to mating results in hypoglycaemia, hypoxia and growth retardation of the fetal sheep (Harding, Jones & Robinson 1985). However, it has also been demonstrated that the efficiency of the placenta, expressed as g fetus produced per g placenta, decreases as placental weight increases (Mellor 1983). In normally nourished ewes, the placenta consumes up to 60% of the glucose that is taken up from the uterine circulation (Owens, Falconer & Robinson 1989). Thus, the larger the placenta in relation to uterine mass and blood supply the greater the proportion of substrates that is likely to be consumed by the placenta rather than be available for growth of the fetus. Placental function continues to develop after placental growth has stopped, with increased exchange surface area, blood flow and substrate transfer capacity (Owens 1991) so placental function is also likely to have an important role in the maintenance of fetal growth in late gestation.

Glucose is the most important metabolic substrate for fetal growth (Battaglia & Meschia 1978). A major source of fetal glucose is glucose from the maternal circulation but other substrates such as amino acids and lactate may also be a resource for fetal glucose production (Hodgson, Mellor & Field 1980). Glucose crosses the placenta by facilitated diffusion and transfer from the mother to the fetus is enhanced by the glucose concentration gradient across the placenta, with maternal blood glucose over twice the concentration of fetal blood glucose. In late gestation a large proportion of glucose from the mother is converted to lactate by the placenta which is then preferentially secreted to the fetus. Because lactate has a low placental permeability the conversion of glucose to lactate allows
carbohydrate to be retained in the fetal circulation. Amino acids are also important substrates and under normal circumstances a far greater proportion of them reach the fetus than is the case for glucose. Amino acids are actively transported across the placenta and this may account for higher fetal blood amino acid concentrations, especially of essential amino acids (Battaglia & Meschia 1978).

The high glucose requirement of placental metabolism means that when glucose supply is severely restricted as in severe maternal undernutrition or starvation amino acids become a more important energy source. The fetus and placenta are able to catabolise amino acids for energy in the rat (Johnson, Dunham, Skipper & Loftfield 1986) and sheep (Liechty, Barone & Nutt 1987; Liechty, Kelly & Lemons 1991). As catabolism continues urea concentrations increase in the fetal circulation (Simmons, Meschia, Makowski & Battaglia 1974; Faichney & White 1987), reflecting amino acid de-amination. It has been suggested that in the above situation branched chain amino acids are preferentially used by the fetus and placenta as an alternate metabolic substrate (Liechty et al. 1991). If substrate restriction becomes more severe fetal tissues may themselves become suppliers of amino acids to the placenta and fetal wasting may occur (Owens, J.A., Owens, P.C. & Robinson 1989). From these studies, the importance of fetal glucose supply in fetal growth is apparent and in addition, the amount of glucose available to the placenta and fetus determines the usage of other substrates such as the amino acids.
INSULIN AND FETAL GROWTH.

Maternal and fetal plasma insulin do not directly influence placental glucose transfer (Urbach, Mor, Ronen & Brandes 1989). Although insulin has no direct effect on placental glucose transport the effect of fetal insulin on fetal glucose consumption may change the transplacental glucose concentration gradient in favour of increased facilitated diffusion of glucose from mother to fetus (Jodarski, Shanahan & Rankin 1985). Insulin is also able to stimulate umbilical uptake of amino acids (Phillips, Rosenkrantz, Lemons, Knox, Porte & Raye 1990) but its role in placental transport of amino acids is not known. Fetal pancreatectomy and resulting hypoinsulinaemia is associated with markedly depressed fetal uptake of substrates (Fowden 1988) and impaired fetal growth (Fowden, Hughes & Comline 1989), while replacement of insulin to the pancreatectomised fetus restores substrate uptake by the fetus and returns growth rate to normal. However, doubling of fetal plasma insulin concentrations does not cause fetal growth rate to increase above normal rates suggesting that the main role of insulin is in promoting substrate uptake and not in the direct regulation of fetal growth (Fowden et al. 1989). Maternal diabetes mellitus leads to fetal hyperglycaemia, hyperinsulinaemia and adiposity of the fetus while fetal hypoinsulinaemia is associated with intrauterine growth retardation (Fowden 1993).

At supraphysiological concentrations insulin can act as a mitogenic factor in many cell types in vitro but it is likely that this action is mediated by the type 1 IGF receptor and not the insulin receptor (Hill & Milner 1985). This suggests that at normal physiological concentrations insulin does not have a very important role as a mitogenic factor in vivo.

Insulin may have an important role in the regulation of the IGFs (Hill & Milner 1985; Gluckman, Butler, Comline & Fowden 1987; Fowden 1993; see section on insulin and IGF regulation).
THE INSULIN-LIKE GROWTH FACTORS (IGFs).

(i) Peptides.

Insulin-like growth factor (IGF)-I, also known as somatomedin-C, is a single chain polypeptide of 70 amino acids and has a molecular mass of 7649 Da (Rinderknecht & Humbel 1978a). IGF-II, also a single chain polypeptide, is composed of 67 amino acids and has a molecular mass of 7471 Da (Rinderknecht & Humbel 1978b). The two peptides have 67% homology and are well conserved between mammalian species (see Sara & Hall 1990 for review). The A and B chains of IGF-I and IGF-II have a high degree of structural homology with proinsulin. However, the C domains of IGF-I and IGF-II are shorter than that of proinsulin. The IGF peptides also have extensions from the carboxyl terminus, termed D domains.

Other forms of the IGFs have been isolated but their physiological significance has yet to be determined. An IGF-II variant of around 10 kDa, termed 'big IGF-II', has been purified from human plasma (Zumstein, Luthi & Humbel 1985) and it appears that the extension corresponds to the E domain portion of the IGF-II precursor molecule proIGF-II. A form of IGF-I, truncated by 3 amino acids has been isolated from human fetal brain tissue and is proposed to have a role in fetal brain development (Sara, Carlsson-Skwirut, Andersson, Hall, Sjörgren, Holmgren & Jömvall 1986). The same truncated IGF-I peptide, des-IGF-I, has been isolated from bovine colostrum (Francis, Read, Ballard, Bagely, Upton, Gravestock & Wallace 1986) and appears to have higher bioactivity than ordinary IGF-I (Francis et al. 1986; Ballard, Francis, Ross, Bagley, May & Wallace 1987) perhaps because of its low binding to the IGFBPs (Szabo, Mottershead, Ballard & Wallace 1988). However, only one form of each IGF have been characterised from fetal serum and these are identical to that found in the adult sheep (Francis, McNeil, Wallace, Ballard & Owens 1989).

(ii) Circulating Concentrations.

Bioassays for somatomedin-like activity were once commonly used to measure IGFs by determining the influence of serum on uptake and incorporation of radiolabelled sulphur into tissue such as cartilage. The main problems of using bioassays to measure somatomedin-like activity is
their low specificity and the potential interference of IGF-binding proteins (IGFBPs) on bioactivity. Plasma concentrations of the IGFs are now commonly measured using radioimmunoassay (RIA) techniques and the methods used in the current studies will be described in Chapter 2. The accuracy of many IGF RIAs may be confounded by the inadequate removal of IGFBPs in plasma or serum samples prior to RIA (Breier, Gallaher & Gluckman 1991). If residual IGFBPs are present in extracts prior to RIA this may mean that labelled ligand may associate with them rather than the larger antibody protein that is selectively precipitated in the final stage of the assay. Because labelled ligand may be lost in this way the apparent quantity of the hormone in a sample may appear greater than it actually is. Another major difficulty with the RIA technique is raising specific antibodies to either IGF-I, IGF-II or insulin while minimising cross-reactivity. In the absence of suitable antibodies different approaches to RIA have been used in the past to measure IGF-II, such as the rat receptor assay where placental membrane preparations have been used (Gluckman & Butler 1983). It is likely that residual IGFBPs in the sample extracts and IGFBPs associated with the membrane surfaces have affected the accuracy of receptor assays.

In the late-gestation fetal sheep plasma IGF-I circulates at concentrations 3-fold lower than maternal concentrations, while fetal plasma IGF-II concentrations are 3-fold higher than maternal concentrations (Gluckman & Butler 1983). As yet data on the perinatal regulation of plasma IGF-II have been of limited quality because of the assays used e.g. rat receptor assay (Gluckman & Butler 1983). Following birth of the lamb plasma IGF-I concentrations rapidly increase to adult concentrations (Gluckman & Butler 1983; Van Vliet, Styne, Kaplan & Grumbach 1983) while plasma IGF-II rapidly falls to adult concentrations (Gluckman & Butler 1983). The rapid postnatal fall in circulating fetal IGF-II concentrations has also been described in the rat (Moses, Nissley, Short, Rechler, White, Knight & Higa 1980). Free IGF-I has been reported to have a half life of 4-10 min in the circulation of the adult sheep (Hodgkinson, Davis, Burleigh, Henderson & Gluckman 1987) and ~12 min in the circulation of the fetal sheep (Bassett, Breier, Hodgkinson, Davis, Henderson & Gluckman 1990a). Free IGF-II has been reported to have a
plasma half-life of ~9 min in the adult sheep (Hodgkinson, Davis, Moore, Henderson & Gluckman 1988), and apparently ~2 min in fetal sheep (Bassett, Breier, Moore & Gluckman 1990b).

(iii) Tissue Concentrations.

Tissues are the source of circulating IGFs and the concentration of IGF-I in various fetal tissues has been studied, but because of assay difficulties knowledge concerning the tissue distribution of IGF-II is much more limited. The developmental patterns seen in the relative abundance of rat plasma IGF-I and IGF-II are also seen in rat fibroblast IGF synthesis (Adams, Nissley, Handwerger & Rechler 1983). IGF-II mRNA is much more abundant than IGF-I mRNA in fetal tissues (Rotwein, Pollock, Watson & Milbrandt 1987; Han, Lund, Lee & D’Ercole 1988) and this is compatible with the relative concentrations of the peptides found in the fetal circulation. The mRNAs for the fetal IGFs appear to be mainly found in connective tissue and cells of mesenchymal origin (Han, D’Ercole & Lund 1987). The highest abundance of mRNA for IGF-II is in the fetal brain (Lund, Moats-Staats, Hynes, Simmons, Jansen, D’Ercole & Van Wyk 1986). Tissue mRNAs from various organs, species and stages of life show a wide variation in size (Daughaday & Rotwein 1989) and the full physiological significance of these variations has yet to be elucidated. There has been recent speculation that the different sizes of the transcripts could relate to the endocrine or paracrine role of the peptides produced (Adamo, Ben-Hur, Roberts & LeRoith 1991; LeRoith & Roberts 1991; Roberts 1992).

Most human fetal tissues have IGF-I concentrations greater than that of the surrounding blood (D’Ercole, Hill, Strain & Underwood 1986). It was reported that fetal liver had the lowest tissue concentrations of IGF-I, but this is in marked contrast to postnatal studies in rats where the highest IGF-I concentrations are found in the liver (D’Ercole, Stiles & Underwood 1984). In hypophysectomised adult rats liver IGF-I concentrations are markedly reduced, but administration of growth hormone (GH) following hypophysectomy quickly returned liver IGF-I concentrations to normal values. The changes in plasma IGF-I concentrations at parturition and the difference in the regulation and tissue production of IGF-I in the fetus and
the adult may thus reflect the different role of GH in IGF-I regulation before and after birth. The significance of GH in fetal IGF regulation will be discussed later.

(iv) IGF Receptors.

IGF receptors in maternal and fetal tissues fall into 2 categories; type 1 and type 2 receptors which are both wide-spread in fetal human and sheep tissues (Owens, Brinsmead, Waters & Thorburn 1980; Sara, Hall, Misaki, Fryklund, Christanson & Wetterberg 1983). Type 1 receptors are 300-350 kDa and preferentially bind IGF-I, IGF-II and insulin in that order. The primary structure of the type 1 receptor shows similarity to the structure of the insulin receptor (Ullrich, Grey, Tam, Yang-Feng, Tsubokawa, Collins, Henzel, Le Bon, Kathuria, Chen, Jacobs, Francke, Ramachandran & Fujita-Yamaguchi 1986). Like the insulin receptor the type 1 receptor is composed of 4 subunits and has an extensive transmembrane domain.

The type 2 IGF receptor is composed of a single polypeptide chain and has a molecular mass of 250 kDa (Morgan, Edmin, Standring, Fried, Smith, Roth & Rutter 1987). The receptor has a large extracellular domain, a single transmembrane region and a small cytoplasmic domain. It has a high binding affinity for IGF-II, a much lower affinity for IGF-I and does not bind insulin (Massaque & Czech 1982; Rechler & Nissley 1985).

Circulating forms of the type 2 receptor have been found in the rat (Kiess, Greenstein, White, Lee, Rechler & Nissley 1987), human (Causin, Waheed, Braulke, Junghans, Maly, Humbel & Von Figura 1988) and sheep (Gelato, Rutherford, Stark & Daniel 1989). The circulating form is smaller and truncated at the carboxyl terminus (MacDonald, Tepper, Clairmont, Perregaux & Czech 1989). It apparently occurs in the circulation because of extracellular release (Clairmont & Czech 1991). The type 2 receptor is present in much greater quantities in the fetal circulation than the maternal circulation and binds much IGF-II (Kiess et al. 1987; Gelato et al. 1989). Studies with rat tissue in culture have shown that there are developmental changes in regulation of receptor release with maximal release occurring in
fetal and neonatal life (Bobbeck, Scott & Baxter 1992). During fetal life the major tissue sources of the released receptor are heart and muscle whereas liver is the major source in the adult rat (Bobbeck et al. 1992). The type 2 receptor will be discussed further later in the Chapter.

(v) The Fetal GH-IGF Axis.

It is clear that GH-stimulated IGF secretion mediates most of the effects of GH on postnatal growth (Schoenle, Zapf, Hauri, Steiner & Froesch 1982; Schoenle, Zapf & Froesch 1982). Early fetal decapitation or hypophysectomy studies suggested that GH had little role in IGF regulation and growth during fetal life (Hill, Davidson & Milner 1979; Gluckman & Butler 1985). However, children with congenital growth hormone deficiency have recently been reported to have reduced birth length in relation to weight (Gluckman, Gunn, Wray, Cutfield, Chatelain, Guilbaud, Ambler, Wilton & Albertsson-Wikland 1992a) and more recent hypophysectomy studies on the sheep fetus in utero demonstrate that fetal plasma IGF-I regulation and growth may have some GH dependence (Mesiano, Young, Hey, Browne & Thorburn 1989). GH receptor mRNA and the binding of GH to tissue GH receptors before birth is much lower than postnatally (Klempt, Bingham, Breier, Baumbach & Gluckman 1993). In addition the negative feedback of circulating IGF-I on GH secretion is limited (Blanchard, Goodyer, Charrier & Barenton 1988) suggesting that GH may have only a small role in IGF-I regulation in fetal life.

(vi) The PL-IGF Axis.

Serum somatomedin concentrations in pregnant rats are unaffected by hypophysectomy (Daughaday & Kapadia 1978) and PL was later suggested to be responsible for maintenance of circulating somatomedin concentrations in the absence of GH (Daughaday, Trivedi & Kapadia 1979). However, the emergence of placental GH variants as potential regulators of maternal endocrine IGF-I during pregnancy (Caufrieze, Frankenne, Englert, Goldstein, Cantraine, Hennen & Copinschi 1990) may suggest that PL is not solely responsible. Purified oPL has been reported to cause increases in circulating IGF-I in hypophysectomised non-pregnant rats (Hurley, D’Ercole, Handwerger, Underwood, Furlanetto & Fellows 1977). Recombinant oPL has similar effects in growth hormone deficient
rats (Singh, Ambler, Breier, Klempt & Gluckman 1992). It should be noted that oPL and rat PL (rPL) have very low sequence homology (Colosi, Thordarson, Hellmiss, Singh, Forsyth, Gluckman & Wood 1989) and therefore the action of oPL on rat plasma IGF-I regulation may not be representative of the actions of rPL. Immunoneutralisation studies in the pregnant ewe argue against a role for PL in IGF regulation (Waters, Oddy, McCloghry, Gluckman, Duplock, Owens & Brinsmead 1985).

As yet there have been few studies that characterise the actions of PL in the fetus but human PL (hPL) has been reported to increase IGF-I release from human fetal hepatocytes and myocytes (Hill, Crace & Milner 1985) and pancreas *in vitro* (Swenne, Hill, Strain & Milner 1987). PL purified from ovine placenta has also been demonstrated to stimulate IGF-II synthesis in rat fibroblasts *in vitro* (Adams *et al.* 1983).

No *in vivo* studies have been reported that test the effect of recombinant PL on plasma IGF regulation in the homologous species.

(vii) Nutritional Regulation of the IGFs.

The postnatal nutritional regulation of circulating IGF-I has been studied extensively. Fasting for 3 d reduces plasma IGF-I in humans but subsequent GH treatment does not cause an increase in IGF-I concentrations suggesting that the relationship between the two hormones has been uncoupled (Merimee, Zapf & Froesch 1982). Studies in the rat suggest that this uncoupling occurs because starvation causes a fall in GH receptor mRNA and subsequently GH receptor number (Straus & Takemoto 1990). Protein restriction in the rat also causes a reduction in circulating IGF-I but not GH receptor number and in this case GH treatment caused circulating IGF-I concentrations to increase (Maiter, Fliesen, Underwood, Maes, Gerard, Davenport & Ketelslegers 1989). The above study suggests that there is post receptor resistance to the regulation of circulating IGF-I by GH (Maiter *et al.* 1989). Caloric content and protein content appear to be equally important in the regulation of circulating IGF-I postnatally (Isely, Underwood & Clemonns 1983; Clemonns, Seek & Underwood 1985).
There has been much less characterisation of the postnatal regulation of circulating IGF-II because of the difficulties in assaying plasma or serum samples for IGF-II. Studies that are available suggest that in the human (Merimee et al. 1982) and the rat (Philipps, Drakenberg, Persson, Sjögren, Eklöf, Hall & Sara 1989) circulating concentrations of IGF-II fall with starvation but not to the same extent as those of IGF-I. The specific nutritional factors that regulate circulating IGF-II are not known.

In the sheep fetus 3 days of maternal starvation resulted in a 30-40% decrease in fetal plasma IGF-I concentrations but subsequent maternal ad libitum refeeding for 48 h fetal plasma caused a return to normal values (Bassett, Oliver, Breier & Gluckman 1990c). Insulin-induced maternal hypoglycaemia resulted in fetal hypoglycaemia and reduced fetal plasma IGF-I and IGF-II concentrations (Owens, J.A., Owens, P., Kind, Smeaton, Ballard & Robinson 1989). Fasting of the pregnant rat also resulted in decreased fetal serum IGF-I concentrations and hepatic IGF-I mRNA content while fetal serum IGF-II concentrations were decreased to a lesser extent with no reduction in fetal hepatic IGF-II mRNA content (Straus et al. 1991). It is not clear from these studies whether circulating fetal IGF concentrations are regulated directly or via maternal factors, or which specific fetal substrates are involved.

The dominant influence of fetal substrate availability on fetal growth has been described previously. As the IGFs are suggested to have important roles in the regulation of fetal growth it is important that their circulating concentrations are regulated by fetal substrate availability so their growth promoting activities will match fetal substrate supply. One of the aims of this thesis is to characterise the regulation of fetal plasma IGFs by specific fetal substrates so that an understanding can be gained of the inter-relationships between fetal substrate supply, the IGFs and fetal growth.

(viii) Insulin and IGF Regulation.

Early studies measuring somatomedin-like bioactivity suggest that fetal hyperinsulinaemia is associated with elevated circulating IGF concentrations (Heinze, Nguyen, Thi, Vetter & Fussgänger 1982; Spencer, Hill, Garsson, MacDonald & Colenbrander 1983; Hill & Milner 1980; Susa
Fetal pancreatectomy is associated with elevated plasma glucose but decreased plasma insulin and IGF-I concentrations (Gluckman et al. 1987). Insulin replacement to adult streptozotocin-diabetic rats returns hepatic IGF-I mRNA expression to normal values in vitro (Böni-Schentzler, Schmid, Meier & Froesch 1991) and in vivo (Pao, Farmer, Begovic, Goldstein, Wu & Phillips 1992) suggesting that insulin has a direct effect on IGF-I mRNA transcription. However, it is possible in these experiments that insulin-stimulated glucose uptake was responsible for the increase in hepatic IGF-I mRNA expression. The possible involvement of insulin in fetal plasma IGF-II regulation has not been investigated. Fetal plasma IGF-II concentrations were reported to increase following fetal pancreatectomy (Gluckman et al. 1987) but the rat receptor assay then used to measure IGF-II may have been unreliable. The effect of insulin on fetal plasma IGF regulation has yet to be fully characterised.

(ix) Actions of the IGFs and Role in Fetal Growth.

Association of the IGFs with Fetal Growth.

Circulating concentrations of IGF-1 have been reported to correlate with birth weight in humans (Gluckman, Johnson-Barrett, Butler, Edgar & Gunn 1983; Ashton, Zapf, Einschenk & MacKenzie 1985) and other animals (Daughaday, Parker, Bawowski, Trevedi & Kapodia 1982; Breier, Gluckman & Bass 1988). However circulating IGF-II concentrations do not correlate with birth weight (Daughaday et al. 1982; Gluckman et al. 1983). Low concentrations of circulating IGF-I and IGF-II are reported in studies where intrauterine growth retardation has been experimentally-induced (Straus et al. 1991; Owens, J.A., Kind, Owens, P.C., Carbone & Robinson 1991). When mouse embryos from lines selected for either high or low plasma IGF-I concentrations are transplanted into an unrelated mouse breed the fetuses from the high IGF-I line grow to be larger than the low IGF-I line suggesting fetal IGF-I is a determinant of fetal growth (Gluckman, Morel, Ambler, Breier, Blair & McCutcheon 1992b). Administration of IGF-I to pregnant mice and rats throughout pregnancy abolishes maternal constraint while placental size remains unaffected suggesting the IGF-I has improved placental performance (Gluckman et al. 1992). All of the above studies suggest that the IGFs play a crucial role in the regulation of fetal growth. However direct testing is still awaited.
Metabolic Effects of the IGFs and Relation to Fetal Growth.

The IGFs exhibit insulin-like effects such as inhibition of lipolysis, stimulation of glucose transport in fat and muscle tissue and stimulation of glycogen synthesis (reviewed by Froesch, Schmid, Schwander & Zapf 1985). However, the IGFs have much lower potency than insulin, especially in adipose tissue, and it appears that most of these activities are mediated by the insulin receptor (Zapf, Schoenle, Waldvogel, Sand & Froesch 1981; King, Kahn, Rechler & Nissley 1980). Recently other studies suggest that IGF-I may indeed have actions on glucose metabolism that are different than insulin and independent of the insulin receptor. Insulin, and IGF-I at a higher dose, have both been observed to increase plasma glucose clearance in the fasted rat (Jacob, Barrett, Plewe, Fagin & Sherwin 1989) and fasted lamb (Douglas, Gluckman, Ball, Breier & Shaw 1991) but unlike insulin, IGF-I failed to suppress glucose production. IGF-II infusion at a similar dose to IGF-I accelerated plasma glucose clearance in the fasted lamb but also had no effect on glucose production (Douglas et al. 1991).

The effects of the IGFs on fetal glucose metabolism have yet to be elucidated.

IGFs may also have a role in protein metabolism. IGF-I inhibits protein breakdown in cells in culture (Ballard, Knowles, Wong, Bodner, Wood & Gunn 1980). IGF-I treatment of starved rats has been reported to limit weight loss (O'Sullivan, Gluckman, Breier, Woodall, Siddique & McCutcheon 1989) and more specifically to inhibit protein breakdown (Jacob, Barrett, Plewe, Fagin & Sherwin 1989). The same effect has been reported in nitrogen restricted rats (Tomas, Knowles, Owens, Read, Chandler, Gargosky & Ballard 1991) and starved lambs (Douglas et al. 1991). At least part of this effect was mediated by the type 1 receptor and there is evidence to suggest that IGF-I, but not IGF-II, promotes protein synthesis (Douglas et al. 1991; Koea, Breier, Shaw & Gluckman 1992). Co-administration of IGF-II in fact blocked the anabolic effect of IGF-I (Koea et al. 1992). The blocking was not due to an effect of IGF-II displacing IGF-I from IGFBPs as total plasma IGF-I was unaffected by IGF-II.
infusion. This may suggest that IGF-II modulates the effect of IGF-I at the type 1 receptor.

Preliminary studies in the sheep fetus suggest that fetal IGF-I infusion decreases amino acid oxidation and increases placental transfer and fetal uptake of amino acids (Harding, Lui & Evans 1992).

IGFs and Growth and Differentiation.

IGF-I and IGF-II have a role in the regulation of cell replication in many cell types (Sara & Hall 1990; Froesch et al. 1985; Gluckman 1986). It has been suggested that the IGFs act as 'progression factors' prior to the DNA synthesis phase of the cell cycle (Stiles, Capone, Sher, Antoniades, Van Wyk & Pledger 1979). The IGFs are also suggested to be important factors in cell differentiation (Sara & Hall 1990). Although there is evidence to suggest that the type 1 IGF receptor mediates the mitogenic and metabolic actions of the IGFs associated with growth the same cannot be said for the membrane bound type 2 receptor. An antibody that blocks the binding of IGF-II to the type 2 receptor failed to affect the growth of rat 18,54 cells as measured by cell replication and uptake of tritiated thymidine (Nissley, Lee & Kiess 1991). These rat cells produce their own IGF-II and exhibit autonomous growth.

Circulating IGF-II concentrations and tissue IGF-II mRNA expression are higher in fetal life than adult life suggesting IGF-II has a more important role in fetal life. Gene targeting has recently been used to disrupt one of the alleles of the IGF-II gene in mouse embryonic stem cells (DeChiara, Efstratiadis & Robertson 1990). The gene disruption caused growth retardation of the heterozygote with much lower body weight, placental weight and much lower IGF-II mRNA tissue expression. These gene disruption experiments suggest that IGF-II has a crucial role in at least early embryonic development. Other investigators have used transplantation of rat embryos and fetal rat paws into the kidney capsule of adult rats as a model to compare the effects of IGF-I and IGF-II in embryonic and fetal development (Nicoll, Liu, Alarid, Chiang & Russel 1991). Infusion of an IGF-I antiserum into the renal artery of the kidneys bearing the transplanted embryo had no effect on growth and development
until the fetal stage of growth was reached at 16 d when the inhibitory effect of the IGF-I antiserum on fetal growth was apparent. Infusion of IGF-I and IGF-II demonstrated that IGF-II was twice as effective as IGF-I in stimulating growth of embryos and paw plants (Nicoll et al. 1991). The rat paw pads had severe impairment of skeletal differentiation when transplanted into hypophysectomised hosts, but this was corrected by infusion of either IGF, confirming that both the IGFs may have roles in tissue differentiation. The gene disruption experiment and embryo and tissue transplant experiments are among the first investigations to provide direct support for the role of the IGFs in fetal growth and development.
I. Introduction.

THE IGF BINDING PROTEINS (IGFBPs).

(i) Characterisation.

There are important differences in the idiotypic regions of the IGFBPs between species and also there are phosphorylated and glycosylated forms of the proteins. Because of this variability, antibodies raised against IGFBPs from one species may not necessarily be suitable for use in the determination of IGFBP concentrations in other species. Unless homology between species is known, individual IGFBPs of a species have to be purified, sequenced and cloned before suitable antibodies, standards and ligands can be produced for analytical techniques such as RIA and associated immuno-electrophoretic methods.

The fact that individual groups have had to develop their own analytical techniques has meant that a large array of nomenclature has been used. Ligand blot analysis uses the natural affinity of the IGFBPs for the IGFs, usually radiolabelled, to detect different bands after gel electrophoresis. The major pitfall in ligand blot analysis is that the different IGFBPs have different binding affinities for each IGF and bands of different IGFBPs can migrate to the same position during gel electrophoresis. Because of the different ability of individual IGFBPs to bind IGFs, ligand blot techniques can only give an indication of the circulating concentration of a given IGFBP. For this reason, where plasma IGFBP data are generated by ligand blot analysis they will be described as plasma levels rather than plasma concentrations. Western immuno-blot techniques use radiolabelled antibodies to specific IGFBPs after gel electrophoresis. The methodologies used for ligand blot and western blot analysis are described in Chapter 2. RIA is often favoured as an analytical technique because it is both a specific and directly quantitative method and large numbers of samples can be analysed in a short time. Unfortunately accurate measurement of functional IGFBPs by RIA could be confounded by the presence of biologically inactive fragments that have the antigenic site. Where western immuno-blot or RIA techniques have been used to measure IGFBPs data will be described as plasma concentrations. In this thesis the nomenclature described by Drop (1989) and Ballard, Baxter, Binoux, Clemmons, Drop, Hall, Hintz, Rechler, Rutanen & Schwander...
(1989) will be used to describe the various forms of IGFBPs. They described 3 IGFBPs, named IGFBP-1, BP-2 and BP-3, but since that publication other forms of IGFBP have been described and one of these has been assigned the name IGFBP-4 (Shimasaki, Uchiyama, Shimonaka & Ling 1990; La Tour, Mohan, Linkhart, Baylink & Strong 1990). The other 2 since purified and cloned are IGFBP-5 (Shimasaki, Shimonaka, Zhang & Ling 1991) and IGFBP-6 (Shimasaki, Gao, Shiminoka & Ling 1991), but as very little is known about their regulation and function only the first 4 IGFBPs will be described in any detail.

(ii) IGFBP-1.

IGFBP-1, also known as placental protein 12 (PP12), is a 25 kDa protein that was first isolated from human maternal and fetal serum collected at birth (Bognar, Than, Csaba & Szabo 1981). The same protein was later isolated and characterised from human amniotic fluid (Póvoa, Enberg, Jörnvall & Hall 1984). Concentrations of IGFBP-1 in the maternal circulation exhibit a diurnal rhythm (Baxter & Cowell 1987), but it is not known whether circulating concentrations of fetal IGFBP-1 have a rhythm. IGFBP-1 has been characterised in maternal and fetal sheep plasma by ligand blot analysis (Gallaher, Breier, Oliver, Harding & Gluckman 1992). IGFBP-1 is secreted by hepatocytes, fibroblasts, uterine epithelium, stroma and decidua and appears to bind IGF-I and IGF-II with equal affinity (Clemmons 1990).

Regulation of BP-1.

Maternal and fetal plasma IGFBP-1 levels as measured by ligand blot increase on 72 h starvation of the pregnant ewe (Gallaher et al. 1992; Osborn, Fowlkes, Han & Freemark 1992) while maternal and fetal hepatic IGFBP-1 mRNA content falls (Osborn et al. 1992). Glucose replacement to ewes at the end of starvation rapidly returns maternal and fetal plasma IGFBP-1 levels and hepatic IGFBP-1 mRNA content to normal (Osborn et al. 1992).

In human fetal liver explants both insulin and glucose are counter-regulators of IGFBP-1 production suggesting that intracellular glucose availability is an important regulator (Lewitt & Baxter 1989). Insulin has
recently been shown to reduce hepatic IGFBP-1 transcription *in vitro* (Unterman, Oehler, Murphy & Lascon 1991) and *in vivo* in streptozotocin diabetic rats (Pao *et al.* 1992). These studies do not exclude the possibility that insulin-induced increases in glucose uptake may influence hepatic IGFBP-1 mRNA transcription. In adult humans it was found that substrates which stimulated hexose uptake by insulin were most effective at depressing plasma IGFBP-1 concentrations (Snyder & Clemmons 1990). However, in the same study plasma IGFBP-1 concentrations were also counter-regulated by substrates that stimulate glycolysis but not insulin-mediated hexose uptake. This suggests that there could be insulin-independent effects of glucose on plasma IGFBP-1 regulation. Further evidence for this hypothesis comes from the *in vitro* observation that adenylate cyclase inhibitors have an additive effect on fetal hepatic IGFBP-1 production to those of hexose-uptake inhibitors (Lewitt & Baxter 1990). Glucose infusion and subsequent increases in circulating insulin concentrations could also result in an insulin-induced increase in transcapillary transfer of IGFBP-1 out of the vasculature, as described in the isolated, perfused rat heart (Bar, Boes, Clemmons, Busby, Sandra, Dake & Both 1990).

**Influence of IGFBP-1 on IGF Activity.**

The ability of insulin to stimulate the transmembrane movement of IGFBP-1 (Bar *et al.* 1990) could indicate that circulating IGFBP-1 may be important in the transfer of IGFs from the vasculature into tissues and perhaps in the targeting of IGFs to paracrine activities. However, it has not been definitively shown that bound IGF accompanies IGFBP-1 across the membrane. IGFBP-1 has been reported to have stimulatory effects (Elgin, Busby & Clemmons 1987) or inhibitory effects (Rutanen, Pekonan & Mäkanin 1988; Ritvos, Ranta, Julkanen, Suikkari, Voutilainen, Bohn & Rutanen 1988; Burch, Correa, Shively & Powell 1990) on IGF mediated activities by modulating the binding of IGFs to type 1 receptors. Cell surface association may account for stimulatory effects of IGFBP-1 while free IGFBP-1 may be more inhibitory (Busby, Hossenlopp, Binoux & Clemmons 1989). This suggests that apart from the equilibrium between circulating IGFs, IGFBPs and receptors there is also the involvement of IGFBPs associated with the cell surface. Small differences in IGFBP-1
structure are thought to determine if cell surface association occurs (Busby et al. 1989). The phosphorylation state of IGFBP-1 is now also thought to be a determinant of the influence of IGFBP-1 on IGF bioactivity (Clemmons 1991).

Because of the rapid responsiveness of circulating IGFBP-1 concentrations to changes in circulating glucose it has been suggested that endocrine IGFBP-1 has an important role in blood glucose regulation by modulating insulin-like effects of the IGFs (Lewitt & Baxter 1991).

(iii) IGFBP-2.

IGFBP-2 is a 34 kDa protein secreted by hepatocytes, decidua and smooth muscle, and has some structural homology with IGFBP-1 (Brown, Chiariotti, Orlowski, Mehlam, Burgess, Ackerman, Bruni & Rechler 1989). IGFBP-2 binds IGF-II with greater affinity than IGF-I (Clemmons 1990). Circulating levels of IGFBP-2 as measured by ligand blot appear to be developmentally regulated with higher levels reported in the fetal circulation (Zapf, Schmid, Guler, Waldvogal, Houri, Futo, Hossenlopp, Binoux, & Froesch 1990; Gallaher et al. 1992). A similar finding has been reported in a study where RIA was used to measure serum IGFBP-2 concentrations (McCusker, Cohick, Busby & Clemmons 1991a).

Regulation of BP-2.

There are conflicting reports on the effect of starvation on maternal and fetal plasma IGFBP-2 levels (Gallaher et al. 1992; Osborn et al. 1992). An increase in plasma IGFBP-2 levels during starvation is difficult to explain because it appears from studies in the adult human that IGF-1 treatment increases plasma IGFBP-2 levels (Zapf et al. 1990). The increase in plasma IGFBP-2 levels during starvation may be in response to changes in metabolic factors other than glucose.

Influence of IGFBP-2 on IGF Activity.

IGFBP-2 is the predominant carrier of IGFs in the fetal circulation (Zapf et al. 1990; McCusker et al. 1991a; Gallaher et al. 1992) and therefore an increase in fetal plasma IGFBP-2 levels on starvation may be important in modulating the insulin-like actions of the IGFs. IGFBP-2 has
also been suggested as a transporter of IGFs across capillary membranes (Bar et al. 1991) but it it is not known whether bound IGF crosses with IGFBP-2. Like IGFBP-1, BP-2 modulates binding of IGFs with the cell surface receptors (Clemmons 1990 & 1991). IGF-I inhibition of proteolysis in L6 myoblasts is blocked by IGFBP-2 interfering with the receptor binding of IGF-I (Ross, Francis, Szabo, Wallace & Ballard 1989).

(iv) IGFBP-3.

IGFBP-3 is a 37-53 kDa protein first characterised by Martin & Baxter (1986). In the circulation IGFBP-3 exists in a ~150 kDa complex in association with an acid labile protein (ALP) of around 88 kDa. Up to 25 % of the molecular mass of IGFBP-3 is composed of carbohydrate. IGFBP-3 binds IGF-II with slightly more affinity than IGF-I and hepatocytes, fibroblasts and endothelium are the sources of secreted IGFBP-3 (Clemmons 1990). IGFBP-3 is the major carrier of circulating IGFs postnatally (Baxter & Martin 1989). Recent reports have suggested that circulating levels of the 150 kDa complex, as measured by ligand blot, decrease in the mother in late gestation and that a serum protease may be responsible for the disappearance (Davenport, Clemmons, Miles, Camacho-Hubner, D’Ercole & Underwood 1990; Hossenlopp, Segovia, Lassare, Roghani, Bredon & Binoux 1990). Suikkari & Baxter (1992) have since reported that circulating IGFBP-3 is functionally normal in late gestation, is in fact present in increasing concentrations in late gestation as measured by RIA and is able to form the 150 kDa complex (Suikkari & Baxter 1992). The earlier studies used ligand blot analysis rather than RIA and it may be possible that harsh treatment of the samples prior to electrophoresis may have altered the IGF binding capability of IGFBP-3.

Regulation of IGFBP-3.

Maternal plasma IGFBP-3 levels fall during 72 h starvation of the pregnant ewe (Gallaher et al. 1992). A fall in maternal plasma IGF-I concentration during starvation may have been responsible since a number of studies using ligand blot analysis suggest that IGF-I regulates plasma IGFBP-3 levels (Zapf et al. 1990; Camacho-Hubner, Clemmons & D’Ercole 1991; Coulsen, Wass, Abdulla, Cotteril & Holly 1991). However, the observation that Laron-type dwarfs have very low circulating IGFBP-3
levels as assessed by ligand blot suggests that growth hormone may be the major influence on BP-3 regulation (Cotterill, Holly, Taylor, Davies, Coulsen, Preece, Wass & Savage 1992). The fact that IGF-I treatment of Laron dwarfs had no effect on plasma IGFBP-3 levels (Rosenfeld, Rosenbloom, Guevara-Aguirre, Berg, Cohen, Diamond, Fielder, Francke, Gargoski, Vaccarello & Wilson 1992) provides further support for the dominant role of GH in plasma IGFBP-3 regulation. The fall in maternal plasma IGFBP-3 levels during starvation (Gallaher et al. 1992) may therefore be due to GH resistance caused by the down regulation of the GH receptor on starvation.

Influence of IGFBP-3 on IGF Activity.

Circulating IGFBP-3 as part of the 150 kDa complex, is the major IGF carrier postnatally (Baxter & Martin 1989). Binoux & Hossenlopp (1988) reported that the 150 kDa complex does not cross the intact capillary membrane, but a recent study suggests that uncomplexed IGFBP-3 can (Boes, Booth, Sandra, Dake, Bergold & Bar 1992). IGFBP-3 has stimulatory effects (Blum, Jenne, Reppin, Kietzmann, Ranke & Bierich 1989) and inhibitory effects (Gopinath, Walton & Etheron 1992) on receptor binding and bioactivity of IGF-I. Like IGFBP-1, BP-3 enhances IGF binding and bioactivity when in association with the cell surface and inhibits binding and bioactivity when free from the surface (Conover 1991). A non-glycosylated form of IGFBP-3 produced in bacteria was demonstrated to have the same capability of stimulating or inhibiting IGF binding and bioactivity suggesting the carbohydrate moiety does not influence surface association of IGFBP-3 (Conover 1991).

IGFBP-1, BP-2 and BP-3 have all been tested in the same cell monolayer system for their ability to competitively bind IGF-I already associated with cell surface-associated IGFBPs or the type 1 IGF receptor (McCusker, Busby, Dehoff, Camacho-Hubner & Clemmons 1991b). All of the added IGFBPs decreased cell surface and receptor binding of radiolabelled IGF-I with IGFBP-3 the most effective and IGFBP-1 the least effective.
(v) **IGFBP-4.**

Not much is known about this 24 kDa protein originally isolated from human osteosarcoma cell lines (Mohan, Bautista, Wegedal & Baylink 1989) and rat serum (Shimonaka, Schroeder, Shimasaki & Ling 1989). The protein has since been cloned and sequenced (Shimasaki et al. 1990; La Tour et al. 1990). In the sheep it appears to circulate in lower levels than the other IGFBPs as determined by ligand blot (Gallaher et al. 1992).

**Regulation of BP-4.**

There are no consistent changes in plasma IGFBP-4 concentrations on starvation, but there are conflicting reports concerning the role of IGF-I in IGFBP-4 regulation postnatally (Ceda, Fielder, Henzel, Lonie, Donovan, Hoffman & Rosenfeld 1991; Neely & Rosenfeld 1992).

**Influence of IGFBP-4 on IGF Activity.**

The influence of IGFBP-4 on IGF bioactivity has only been investigated in a few studies but it would seem that it has inhibitory influences (Mohan et al. 1989; Shimonaka et al. 1989). Bar et al. (1991) report that insulin caused a decrease in endothelial cell IGFBP (ECBP) in cardiac tissue and release of ECBP into the perfused vasculature of the isolated rat heart. ECBP has since been identified as IGFBP-4 (Boes et al. 1992).

(vi) **Circulating Type 2 IGF Receptor.**

**Regulation of Circulating Type 2 IGF Receptor.**

Very little is known about the regulation of the circulating concentrations of the type 2 receptor. As discussed earlier receptor release from the plasma membrane appears to be developmentally regulated with more occurring during fetal life (Bobek et al. 1992). Insulin has been reported to stimulate the cellular translocation and release of the receptor postnatally (Clairmont & Czech 1990; 1991) but its effects on the regulation of the receptor during fetal life are not known.
Influence of Circulating Type-2 IGF Receptor on IGF Activity.

The high concentration of circulating IGF-II in fetal life (Gluckman & Butler 1983; Van Vliet et al. 1983), the developmental regulation of the circulating type 2 receptor (Bobeck et al. 1992) and the large amount of IGF-II associated with the circulating type 2 receptor (Gelato et al. 1989; Gallaher et al. 1992) suggest the major role of the circulating type 2 receptor in fetal life is as a carrier protein. At present no studies strongly support a stimulatory or inhibitory effect of the circulating type 2 receptor on IGF-II bioactivity.

(vii) Plasma Half-Lives of IGFBPs.

In the only sheep studies reporting plasma half-lives, the IGFBPs were measured by gel chromatography as 150 kDa or 50 kDa forms. This means that uncomplexed forms of some IGFBPs such as IGFBP-3 may elute in the 50 kDa fraction. In the ewe, the 50 kDa form that represents IGFBP-1, BP-2 uncomplexed BP-3 and BP-4 had a considerably shorter plasma half-life than the 150 kDa form that represents the IGFBP-3/ALP complex (93 ± 15 min and 306 ± 37 min respectively, Hodgkinson et al. 1987). In the sheep fetus a large 250 kDa complex, perhaps representing the circulating type 2 receptor, was reported to have a plasma half-life of 385.9 ± 65.4 min, while the 150 kDa and 50 kDa had plasma half-lives of 385.9 ± 65.4 min and 308.0 ± 65.0 min respectively (Bassett et al. 1990b).

(viii) Role of IGFBPs and Circulating type 2 Receptor in Fetal Growth.

The regulation and function of the circulating IGFBPs and type 2 receptor in fetal life have not been investigated to any great extent. Extrapolation from postnatal studies would suggest each IGFBP has similar roles in fetal and postnatal life, although the observation that individual IGFBPs and the type 2 receptor are present in different amounts in the fetal and maternal circulation may suggest otherwise. IGFBP-1 is considered to have a inhibitory role in modulating insulin-like IGF activity (Lewitt & Baxter 1991) and what studies are available from animal models (Straus et al. 1991) and clinical observations (Howell, Perry, Choglay, Bohn & Chard 1985; Wang, Lim, English, Irvine & Chard 1991) may
suggest that the elevated concentrations of circulating IGFBP-1 in the blood of growth retarded newborn are supportive of this inhibitory role. The increase in plasma IGFBP-1 and IGFBP-2 levels on maternal starvation (Gallaher et al. 1992) could also be important in inhibiting insulin-like activities of the IGFs and this may be especially true for IGFBP-2 considering its circulating levels are elevated in fetal life. There are no \textit{in vivo} observations that could suggest association of other IGFBPs with the regulation of fetal growth as yet, but like IGFBP-3, all are important carriers of circulating IGFs and this is especially true for IGFBP-2 and the circulating type 2 receptor during fetal life.

The nutritional regulation of the IGFs and the possible relationship with fetal substrate supply have already been discussed. Nutritional regulation of IGF activity may also occur by the influence of nutrition on the circulating concentrations of the IGFBPs and type 2 IGF receptor. This dual control of IGF activity may be especially important in late gestation when fetal substrate supply is more likely to be restricted.
PLACENTAL LACTOGEN (PL).

(i) Overview.

At the start of the chapter the relationship between fetal substrate supply and the regulation of fetal growth was discussed. Circulating concentrations of insulin and the IGFs appear to be nutritionally regulated in fetal life and may be involved in the interaction between fetal substrate supply and fetal growth. Similarly PL appears to be nutritionally regulated and may have influences on maternal and fetal metabolism that promote fetal growth. In addition, PL may be an important regulator of the IGFs in fetal life.

(ii) Peptide.

As its name suggests PL, also known as chorionic somatomammotropin (CS), is a pregnancy-specific hormone produced by placental tissues and is a member of the lactogenic family of hormones that also include GH and prolactin (PRL). Fully purified oPL is a 198 amino acid protein with a molecular mass of 22 kDa (Colosi et al. 1989; Warren, Liang, Krivi, Siegel & Anthony 1990). Human PL (hPL) and oPL have very low structural homology (25%) as do rodent PL and oPL (< 30%, Colosi et al. 1989). Ovine PL also has low homology with the other members of the lactogenic family, oGH (28%) and oPRL (49%). Ovine PL and human GH (hGH) have low structural homology (26%) but despite this fact oPL competes effectively with hGH for human GH receptor binding (Colosi et al. 1989). Chan, Nie & Pang (1990) have used a monoclonal antibody to oPL to immunohistochemically localise oPL to the uninucleate cells of the ovine fetal trophoblast.

(iii) Circulating Levels.

Early ontogeny studies in the sheep suggest that the fetal/maternal ratio of circulating oPL decreases through gestation, being around 1:1 before 100 d gestation and 1:10 at term or 147 d gestation (Gluckman, Kaplan, Rudolf & Grumbach 1979). Similar ratios have been reported in late-gestation (120-140 d) ewes and fetuses (Handwerger, Crenshaw, Maurer, Barrett, Hurley, Golander & Fellows 1977; Taylor, Jenkin, Robinson, Thorburn, Friesen & Chan 1980). In rodents and humans PL has been reported to circulate in association with macroglobulins (Southard &
I. Introduction.

But similar PL-macroglobulin associations have not been reported in sheep.

(iv) Receptors.

The existence of a distinct PL receptor during pregnancy is a subject of debate. A distinct PL receptor has been reported in fetal and maternal sheep liver that binds oPL with higher affinity than oGH (Freemark, Comer, Korner & Handwerger 1987; Freemark, Comer & Korner 1988; Fowlkes & Freemark 1992). A second class of hepatic receptor that binds oPL and oGH with similar affinity was also suggested but the receptor with higher affinity for oPL was reported to be more prevalent in the fetal sheep liver. However, other studies have since demonstrated that oGH receptor-transfected cell preparations have similar binding characteristics to the proposed PL receptor despite the fact that only oGH receptors are present (Breier, Klempt, Baumbach, Bingham, Gluckman & Skinner 1992). Recently the binding of oGH and oPL to fetal and maternal hepatic tissue throughout gestation has been characterised (Klempt et al. 1993). The ontogeny of oGH and oPL binding and oGH receptor mRNA in fetal and maternal liver exhibit parallel changes throughout gestation suggesting oPL is consistently a stronger binding ligand. These studies argue against a specific PL receptor.

(v) Nutritional Influences on PL.

Nutritional deprivation has been reported to increase circulating concentrations of PL in pregnant women (Tyson, Austin & Farinholt 1971; Kim & Felig 1971), sheep (Brinsmead, Bancroft, Thorburn & Waters 1981) and rats (Fielder, Ogren, Edwards & Talamantes 1987). However, other studies in the 72-h starved ewe report no significant change in maternal plasma PL concentrations (Butler, Huyl, Grandis & Handwerger 1987). In pregnant women hyperglycaemia induced by glucose infusion caused a fall and insulin-induced hypoglycaemia a rise in maternal plasma PL concentrations (Gaspard, Sandront & Luyckx 1974) suggesting an inverse relationship between circulating glucose and PL concentrations.

In the sheep, fetal plasma PL concentrations have been reported to increase on 72 h maternal starvation (Freemark, Comer, Mularoni,
D’Ercole, Grandis & Kodack 1989) and after a longer period of maternal undernutrition (Brinsmead et al. 1981). The increase was accompanied by a fall in hepatic PL receptor numbers and in fetal plasma glucose concentration (Freemark et al. 1989). The same investigators have since infused ewes with glucose after 72 h starvation at a rates designed to restore normal plasma glucose concentrations and have reported that fetal plasma PL fell to pre-starved concentrations, while fetal liver PL receptor number also returned to pre-starved numbers (Freemark, Keen, Fowlkes, Mularoni, Comer, Grandis & Kodac 1992). These studies suggest that fetal plasma PL and glucose concentrations are inversely related, as are fetal liver PL receptor number and fetal plasma PL concentration, while fetal plasma glucose concentration and fetal liver PL receptor number are positively related. The authors suggest that fetal glucose supply regulates fetal plasma PL concentration, and in turn fetal plasma PL regulates fetal liver PL receptor number (Freemark et al. 1992).

Infusion of arginine (Handwerger, Crenshaw, Lansing, Golunder, Hurley & Fellows 1978) and ornithine (Handwerger, Grandis, Barry & Crenshaw 1981) is reported to increase plasma PL concentrations in the ewe. There are no studies reporting the effects of amino acid infusion to the fetus.

High density lipoproteins (HDL) stimulate PL secretion in human trophoblast cells possibly via HDL specific receptors (Handwerger, Quarfordt, Barrett & Harman 1987). Delipidation and proteolysis studies suggest that the protein portion of the HDL is responsible for the PL secretory effect (Handwerger et al. 1987). In vivo HDL has been demonstrated to elevate plasma PL concentrations in the ewe but not the fetus (Grandis, Jorgensen, Kodack, Quarfordt & Handwerger 1989). The effect of HDL on PL release is reported to be mediated via cAMP accumulation (Wu, Jorgensen & Handwerger 1988).

(vi) Actions of PL and Role in Fetal Growth.

Association of PL with Fetal Growth.

A number of studies have reported that there are strong correlations between fetal birth weight and maternal plasma PL concentrations.
(Tayloret al. 1980; Langhoff-Roos, Wibell, Gibre-Medhin & Lindmark 1989), while others have shown weaker correlations between fetal birth weight and fetal plasma PL concentrations (Houghton, Shackelton, Obiekwe & Chard 1984; Hill, Freemark, Strain, Handwerger & Milner 1988). The above correlations may actually reflect the relationship between fetal size, placental size and plasma PL concentrations since PL is solely produced in the placenta. Very few studies have actually studied the relationship between placental mass and circulating PL concentrations but positive correlations have been reported (Falconer, Owens, Allotta & Robinson 1985).

There is no direct evidence for a role of PL in fetal growth and indeed there have been reports of human pregnancies where the gene for PL is missing and fetal growth has not been retarded (Nielsen, Pederson & Kampmann 1979). However, as high concentrations of GH and prolactin are present in the fetal circulation it is possible that the system has a high degree of redundancy and that other lactogenic hormones may fulfill the role of PL in its absence.

**PL and IGF Regulation.**

The literature regarding the suggested role of PL in IGF regulation has already been reviewed. Recombinant PL has yet to be tested *in vivo* for its effect on circulating IGFs in the homologous species.

**PL and Cell Replication.**

Ovine PL has been reported to stimulate ornithine decarboxylase (ODC) activity in the fetal rat liver while oGH, rGH, oPRL and rPRL were without effect (Hurley, Kuhn, Schanberg & Handwerger 1980). ODC activity is a rate limiting step in the synthesis of the polyamines which are themselves important in the regulation of amino acid, protein, RNA and DNA synthesis. In human fetal fibroblasts and myoblasts hPL, but not hGH, caused an increase in the rate of thymidine uptake, suggesting increased DNA synthesis (Hill, Crace & Milner 1985). In a later study it was found that the inclusion of an antibody to IGF-I partially inhibited the effect of hPL on thymidine uptake which suggests at least part of the effect
of the hPL could be attributed to hPL-stimulated IGF-I release (Hill, Crace, Strain & Milner 1986).

**Metabolic Effects of PL.**

PL is postulated to have lipolytic and proteolytic activities in the pregnant mother. Infusion of relatively crude PL-containing extracts into non-pregnant sheep has produced conflicting results. Significant increases in plasma glucose, free fatty acids (FFAs) and urea concentrations have been reported during infusion of PL containing extracts (Thordarson, McDowell, Smith, Iley & Forsyth 1987), suggesting PL has substrate-releasing activities, while an earlier study produced the opposite effect with plasma glucose, FFAs and amino nitrogen (AN) concentrations falling markedly (Handwerger, Fellows, Crenshaw, Hurley, Barrett & Maurer 1976). In vitro studies in mouse fat cells have also yielded conflicting results (Febler, Zaragoza, Benuzzi-Badoni & Genazzani 1972; Fielder & Talamantes 1987). Immunoneutralisation experiments in the ewe failed to cause any significant changes in FFA metabolism although it was reported that maternal plasma FFA concentrations had fallen slightly by the end of the anti-PL antibody infusion (Waters et al. 1985).

PL has been reported to induce insulin resistance in rat adipocytes by an unknown post receptor effect since PL did not interfere with the binding of insulin to the cells (Ryan & Enns 1988).

In fetal rat muscle oPL, but not oGH, has been reported to stimulate amino acid transport (Freemark & Handwerger 1983). Similarly hPL caused increased amino acid uptake in fetal rat myoblasts and fibroblasts (Hill et al. 1986). In these experiments hPL also stimulated the release of IGF-I, but addition of an IGF-I antibody only partially decreased the rate of hPL-stimulated amino acid uptake (Hill et al. 1986).

Freemark & Handwerger (1984) reported that oPL stimulated glycogen synthesis in fetal rat hepatocytes and later showed that oPL also inhibited glucagon-induced glycogen breakdown (Freemark & Handwerger 1985). The effect of oPL on glycogen synthesis has since been reported in
fetal sheep hepatocytes where the activity was reported to be mediated by the putative oPL receptors (Freemark & Handwerger 1986).

Interaction of Nutrition and PL in Fetal Growth.

The evidence for a role of PL in fetal growth is mostly based on in vitro data and by association. Plasma PL concentrations appear to be responsive to changes in nutrition in both the mother and fetus. However, both catabolic and anabolic actions have been described for PL. Elucidation of specific roles for PL in fetal metabolism await further investigation.
OBJECTIVES.

Fetal substrate supply is the dominant influence on fetal growth in late gestation. There is evidence to suggest that the IGFs and PL have roles in the regulation of fetal growth (Gluckman et al. 1990; Handwerger 1991; Owens 1991; Gluckman & Harding 1992). Fetal plasma IGF-I and PL concentrations are influenced by changes in maternal nutrition (Bassett et al. 1990c; Freemark et al. 1987). However, the nutritional regulation of fetal plasma IGF-II and IGFBP concentrations is not clear. The main aim of this thesis is to characterise the nutritional regulation of the IGFs, IGFBPs and PL in the fetal circulation. This information will further elucidate the functional relationship between fetal substrate supply and IGF-I, IGF-II and PL in the regulation of fetal growth in late gestation.

An earlier study using a starvation refeeding model demonstrated that fetal plasma IGF-I concentrations were influenced by maternal nutrition (Bassett et al. 1990c). What was not apparent from this study was which maternal or fetal factors were responsible. We will investigate the role of specific substrates (glucose, total amino acids) and hormones (insulin) in the nutritional regulation of fetal plasma IGF-I, IGF-II and PL concentrations.

The influence of fetal glucose and insulin on fetal plasma levels of the IGFBPs will be determined using ligand blot. It is largely unknown whether the nutritional regulation of fetal plasma IGFBP levels is different in fetal life compared to postnatal life. Nutritional influences on circulating IGFBP levels and hence the bioavailability and bioactivity of the IGFs themselves, may be especially relevant in late gestation when fetal substrate supply is more likely to be restricted. The abundance of the circulating form of the type 2 receptor seems to be unique to fetal life and as yet there have been no studies into its nutritional regulation. Characterisation of the nutritional regulation of circulating type 2 receptor concentrations may be very important given the role IGF-II is suggested to have in the regulation of fetal growth and development.
It is clear from the experiments of Freemark et al. that starvation of the pregnant ewe has significant effects on the regulation of fetal plasma PL concentrations. However the effect of the prior nutritional status of the mother on fetal plasma PL responses to maternal starvation has not been previously described. PL has been postulated to be 'the fetal growth hormone' and a number of studies have shown some influence of PL on fetal IGF-I regulation in vitro (Hill et al. 1985; Swenne et al. 1987). However, as yet highly purified or recombinant PL has not been tested for effects on fetal plasma IGF regulation in the homologous species. We have designed a protocol to test the effect of recombinant oPL on the circulating concentrations of IGF-I, IGF-II and metabolites in the late gestation sheep fetus.

CHAPTER 2. MATERIALS AND METHODS.

ANIMAL MANAGEMENT.

Coopworth-Border sheep were bred at Ruakura Animal Research Station where a progesterone-based synchronised breeding technique was used. After progesterone treatment had been withdrawn from the ewes rams were allowed access to them. Subsequent mating was determined by inspection for markings on ewes from crayon positioned in the ram harness. Pregnancy was confirmed later by ultrasound scan. Ewes were transported to our laboratory at least 2 weeks before surgery to allow acclimatisation to their surroundings. They were fed a standard diet of sheep nuts (NRM, Auckland, NZ) and ad libitum barley straw for bulk. The amount of water consumed has an important influence on the efficiency of ruminant digestion and so was always supplied in excess. Before their arrival in Auckland ewes had exposure to the sheep nuts and experience of indoor conditions. Generally, the ewes ate at least 1200 g of nuts each day while in our laboratory. The sheep nuts used are specifically designed for ruminants and at the above daily intake provide adequate carbohydrate and degradable protein for the energy and nitrogen requirements of the rumen microbial population. The microbial environment of the rumen is particularly sensitive to drastic change in carbohydrate and protein intake so after relatively short term feed restriction marked changes in microbial populations may occur. Upon refeeding there may be some lag before the original microbial environment and level of nutrient uptake is regained. For this reason daily feed intakes were recorded before and during experiments and if ewes failed to eat a consistent amount of feed at a high enough level they were not used in experiments. The body condition of the ewe may indicate what the longer term nutritional state of the ewe has been, so in later experiments body point scores were recorded as described by Mellor (1987). Scoring was on a scale of 1 to 5, 1 being extremely poor and 5 being excellent. Ewes with a score below 3 were excluded from later experiments. Maternal blood glucose was also monitored after surgery, before and during experimentation to determine nutritional state. Even though adult sheep rely on free fatty acid metabolism maternal glucose was a useful measure to use in these studies as glucose is the most readily available and important substrate to the sheep fetus.
After the two weeks acclimatisation fetal surgery was performed at approximately 120 d gestation while the ewes were under halothane anaesthesia. The tarsal veins and arteries of both fetal hind limbs were catheterised. Maternal catheters were also placed in the left carotid artery and jugular vein. After surgery the ewes were placed in individual metabolic cages and allowed at least 5 days to recover before experimentation started. Ewes were given 2.5 ml Streptopen (Pitman-Moore Ltd., Upper Hutt, NZ) intramuscularly prior to, and for 3 days after surgery. Eighty milligrams Gentamycin (Roussel Pharmaceuticals Pty. Ltd., Castle Hill, NSW, Australia) and a penicillin (500 - 1000 mg) were administered intravenously to the fetus for 3 days after surgery. The fetal and maternal catheters were flushed with heparinised saline (10 U/ml) for the first three days after surgery and then on alternate days to maintain catheter patency.

### Table 2a. Fetal and Placental Morphometric Measurements at Post Mortem (134.6 ± 2.9 d gestation).

<table>
<thead>
<tr>
<th>Weights (g)</th>
<th>Mean ± SD</th>
<th>Coeff. Var.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetus</td>
<td>3979.8 ± 814.8</td>
<td>20.5</td>
</tr>
<tr>
<td>Brain</td>
<td>47.8 ± 4.3</td>
<td>9.1</td>
</tr>
<tr>
<td>Thyroid</td>
<td>1.3 ± 0.6</td>
<td>43.5</td>
</tr>
<tr>
<td>Thymus (neck)</td>
<td>9.3 ± 3.0</td>
<td>32.4</td>
</tr>
<tr>
<td>Thymus (chest)</td>
<td>4.6 ± 1.9</td>
<td>40.7</td>
</tr>
<tr>
<td>Heart</td>
<td>31.1 ± 7.1</td>
<td>22.7</td>
</tr>
<tr>
<td>Lung</td>
<td>108.8 ± 20.3</td>
<td>18.6</td>
</tr>
<tr>
<td>Liver</td>
<td>128.0 ± 29.8</td>
<td>23.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>7.4 ± 2.3</td>
<td>30.5</td>
</tr>
<tr>
<td>Adrenals</td>
<td>0.5 ± 0.2</td>
<td>44.7</td>
</tr>
<tr>
<td>Kidneys</td>
<td>27.8 ± 9.2</td>
<td>33.2</td>
</tr>
<tr>
<td>Uterus</td>
<td>560.1 ± 106.4</td>
<td>19.0</td>
</tr>
<tr>
<td>Placental Membranes</td>
<td>262.6 ± 88.1</td>
<td>33.6</td>
</tr>
<tr>
<td>Placentomes (total)</td>
<td>401.8 ± 103.6</td>
<td>25.8</td>
</tr>
<tr>
<td>Mean Placentome</td>
<td>5.4 ± 1.6</td>
<td>30.0</td>
</tr>
<tr>
<td>CRL (mm)</td>
<td>474 ± 36</td>
<td>7.5</td>
</tr>
<tr>
<td>Hind Limb (mm)</td>
<td>347 ± 35</td>
<td>10.2</td>
</tr>
<tr>
<td>Placentome Number</td>
<td>77.2 ± 14.7</td>
<td>19.0</td>
</tr>
</tbody>
</table>

Coeff. Var., coefficient of variation; CRL, crown rump length. (n = 36)

During experimentation care was taken that no more than 10 % of fetal blood volume was removed on a given day. In most experiments, well under this amount of blood was taken. Blood gases (measured on Radiometer Acid-Base Laboratory, Copenhagen,
Denmark), haemoglobin, oxygen saturation (Radiometer Hemoximeter) and concentrations of blood glucose and lactate (YSI Model 2300 STAT Glucose and L-Lactate Analyzer, Yellow Springs Instrument Co. Inc., Ohio, USA) were measured with most samples so fetal well-being could be assessed. Blood was collected in heparinised syringes and placed on ice until measurement of gases or glucose and lactate. The remaining blood was centrifuged at 3000 g for 10 min at 4°C. Plasma was then divided into several aliquots and frozen at -20°C until analysis. For later measurement of glucose, amino nitrogen (AN) and lactate a portion of blood was immediately deproteinised on ice and the supernatants stored at -80°C.

At the completion of an experiment the ewe was killed with an overdose of pentobarbitone and the uterus and its contents weighed and measured. Post-mortem data from all fetuses is grouped as there were no significant differences in fetal or placental parameters between experimental groups (Table 2a). All animal studies were approved by the University of Auckland Animal Ethics Committee.

(i) Metabolite Assays.

Concentrations of metabolites were measured in whole blood in these studies. The main reason for measuring blood values rather than plasma was one of practicality given that we had a YSI Glucose and L-Lactate Analyzer and other analytical methods available that use whole blood. Metabolite assay techniques using whole blood were favoured in our laboratory as the resulting blood concentrations were used with blood flow measurements to calculate placental uptakes. The YSI Analyzer uses very small volumes of blood and provides a reading within 2 min which is especially useful in experiments where blood metabolites are directly manipulated to achieve a desired concentration. However, plasma concentrations of metabolites may have provided a clearer representation of substrates available to tissues given that intracellular metabolites in blood cells may be less available.

Blood glucose and lactate concentrations were measured within 10 min of collection with a YSI Glucose and L-Lactate Analyzer, or when the analyzer was not available, using standard enzymatic micromethods modified for 96-well micro plates (Bergmeyer 1983; Ashour, Gee & Hammock 1987). The values obtained from the YSI analyser and microplate method correlated well for glucose (Fig. 2a) and lactate (Fig. 2b.), and paired values from either method were within 0.05 mM of each other. Blood AN concentrations were measured by the naphthoquinone method (Frame, Russell & Wilhelmi 1943) which was modified in our laboratory to use smaller volumes of blood and reagents (Evans, Ffolliott-Powell & Harding 1992).

(ii) IGF-I Assay.

Plasma IGF-I concentrations were measured by double antibody RIA using rabbit antiserum to recombinant human IGF-I (878/4) at a final titre of 1:250,000 (Breier, Gallaher & Gluckman 1991). The
Correlation of Blood Glucose Analysis Methods.

Figure 2a. Correlation of blood glucose concentrations obtained by YSI Glucose and L-Lactate Analyzer and microplate methods.

antiserum has a cross reaction with IGF-II and insulin of < 0.05% and < 0.001% respectively. In this assay system the minimal detectable dose was 0.06 ng/tube and the half displacement dose was 0.40 ng/tube. Recombinant human IGF-I (rhIGF-I, Ciba, Basel, Switzerland) was iodinated by a chloramine-T method and then purified by exclusion chromatography on a pre-albuminated Sephadex G-75 column (Pharmacia, Uppsala, Sweden, 10 X 600 mm). The eluant used was 0.01 M phosphate buffered saline (PBS, pH 6.2) containing 0.1% gelatin (w/v). Fractions corresponding to the IGF-I monomer were pooled and stored at 4°C until use. The specific activity of $^{125}$I-labelled rh IGF-I was 140 μCi/μg.
Correlation of Blood Lactate Analysis Methods.

Before RIA, plasma samples were subjected to acid-ethanol cryo-precipitation extraction (Breier et al. 1991). The incubation mixture consisted of 100 μl diluted neutralised acid-ethanol solution containing the standard or test plasma and 200 μl of assay buffer (0.15 M NaCl and 0.01 M phosphate, pH 7.6, containing 0.1% bovine serum albumin (BSA) and 0.33% EDTA) containing IGF-I antiserum. After pre-incubation for 1 h at room temperature, $^{125}$I-labelled rhIGF-I (20,000 cpm/tube) was added in 200 μl of assay buffer and the incubation was continued for 18-24 h at 4°C. Bound and free IGF-I were separated by addition of 1 ml of a pre-precipitated second antibody containing 0.01 M PBS, 1% (v/v) sheep anti rabbit gammaglobulin, 0.1 % (v/v) normal rabbit serum and 5% (w/v) polyethylene glycol 6000 (PEG 6000). The test tubes were then incubated for 1 h at room temperature and centrifuged at 3000 g for 30 min at 4°C. The supernatant was decanted and the radioactivity of the remaining pellet was determined using a

We discovered during our validation procedure that some fetal samples from animals undergoing starvation failed to displace parallel to the standard curve after the acid-ethanol cryo-precipitation. Residual IGFBP-2 is markedly elevated during fetal starvation (Gallaher et al. 1992) and failure to remove this binding protein during extraction caused the loss of parallel displacement. Since our antisera (878/4) has very low cross-reactivity with IGF-II we assessed whether added IGF-II could be used to displace the IGF-I from any IGFBPs remaining in the extract. After the cryo-precipitation step, 25 ng/tube recombinant human IGF-II (batch #099EM9, Eli Lilly and Company, Indianapolis, Indiana, USA) was added to the assay buffer (Breier, Blum, Schwander, Gallaher, Milsom & Gluckman 1993). Using this system, the recovery of IGF-I added to fetal plasma before extraction was 85.0 ± 8.9% (n=10) and maternal plasma 84.2 ± 7.6% (n=7). Selected fetal and maternal samples obtained during control, starvation and refeeding periods were also subjected to acid gel chromatography on a Sephadex G-75 column (Pharmacia, Uppsala, Sweden) prior to IGF-I RIA as an alternative method of removing the interfering binding proteins (Breier et al. 1991). The plasma IGF-I concentrations obtained after the acid gel chromatography showed a good correlation with plasma IGF-I concentrations obtained after the cryo-precipitation and IGF-II addition procedures (Fig. 2c). The intra-assay coefficient of variation was 5.0% and the inter-assay coefficient of variation was 9.8%. Plasma IGF-I concentrations are expressed in terms of the international reference preparation (ORR oIGF-I, batch 87/518).

(iii) IGF-II Assay.

Plasma samples were analysed for IGF-II by RIA after acid chromatography (Koea et al. 1992). Recombinant human IGF-II (rhIGF-II, Eli Lilly and Company, Indianapolis, Indiana, USA) was iodinated and the labelled peptide purified using an identical technique to that used for preparation of IGF-I tracer. The specific

Correlation of IGF-I Assay Methods.

![Graph showing correlation between plasma IGF-I concentrations (ng/ml) obtained by RIA after cryoprecipitation and IGF-II addition or G-75 chromatography.]

\[ y = 0.712x + 14.818, \text{R-squared:} \ 0.707 \]

Figure 2c. Correlation between plasma IGF-I concentrations (ng/ml) obtained by RIA after cryoprecipitation and IGF-II addition or G-75 chromatography.

Activity of the $^{125}$I-labelled rhIGF-II tracer was 70 μCi/μg. Plasma (100-200 μl) was acidified with formic acid to a final concentration of 1 M and incubated for 15 min to dissociate IGF-II from its IGFBPs. The acidified serum was then chromatographed on Sephadex G-75 (Pharmacia, Uppsala, Sweden) using a glass column (10 X 900 mm) to separate IGFBPs and IGFs. The column was eluted with 0.25 M formic acid at a flow rate of 0.5 ml/min and 1 ml fractions were collected into silicone-coated glass tubes (Coatasil; Ajax Chemicals Pty., Auburn, Australia) containing 50 μl 1% BSA in distilled water. The column was calibrated with Dextran Blue, $^{125}$I-labelled rhIGF-II and $^{125}$I, and the volume of the eluate containing the native IGF-II was collected and lyophilised. The lyophilised fractions were reconstituted in assay buffer before further analysis. Recoveries of $^{125}$I-labelled rhIGF-II added to plasma were 90.7 ± 5.8 % (n=7).
IGF-II was measured by double antibody RIA (Koea et al. 1992) using a monoclonal anti-rat IGF-II antibody (Amano Pharmaceutical Co. Ltd. Nagoya, Japan) at a final titre of 1:300,000. The antiserum has a cross reaction with IGF-I and insulin of < 1.0% and < 0.01% respectively. The incubation mixture consisted of 100 μl of the reconstituted G-75 extracts (diluted in assay buffer to 1:200 for adult samples, 1:300 for fetal samples), 200 μl of assay buffer containing IGF-II antiserum and 200 μl of assay buffer containing \(^{125}\text{I}\)-labelled rhIGF-II (20,000 cpm/tube). The tubes were incubated for 18-24 h at 4°C. Bound and free IGF-I were separated by addition of 1 ml of a pre-precipitated second antibody containing 0.01 M PBS, 0.1% (w/v) goat anti-mouse gammaglobulin (Sigma, St Louis, Missouri, USA), 0.025% (v/v) normal mouse serum and 5% (w/v) PEG 6000. The tubes were then incubated for 2-3 h at 4°C then centrifuged at 3000 g for 30 min at 4°C. After the supernatant was decanted off the radioactivity of the remaining pellet was determined using the Cobra Auto Gamma counter.

(iv) Insulin Assay.

Antibodies to ovine insulin (Novo, Denmark) were raised in guinea pigs. Recombinant human insulin (rh-Insulin, Eli Lilly and Company, Indianapolis, Indiana, USA) was iodinated and purified using a method identical to that used for the iodination of IGF-I. The specific activity of the \(^{125}\text{I}\)-labelled insulin was 140 μCi/μg. Previous experiments had shown that the use of human recombinant insulin, a more readily available molecule, produced parallel displacement curves to those obtained using sheep insulin. The same assay buffer used in the IGF-I RIA was used in the insulin RIA. The insulin standards and samples were added to each tube, diluted in assay buffer to a volume of 100 μl and then 200 μl guinea pig anti-sheep anti-serum (GC4) was added to give a final tube antibody concentration of 1:200,000. After 24 h incubation at room temperature \(^{125}\text{I}\)-rh-insulin was added to the tubes (25,000 cpm/tube) and incubation continued for another 24 h at 4°C. The separation of bound and unbound tracer was achieved by a 4 h incubation of the tubes with 1 ml of a pre-precipitated second antibody mixture containing 0.01 M PBS, 0.5% sheep anti-guinea pig gammaglobulin (v/v), 0.05% normal guinea pig serum (v/v) and 4% PEG 6000 (w/v). The tubes were centrifuged and counted under the
same conditions as the IGF-I and IGF-II RIAs. The cross reactivity with IGF-I and IGF-II was less than 0.01%. The within assay coefficient of variation was 6.7% and the inter-assay coefficient of variation was 11.5%.

(v) IGFBPs.

Plasma IGFBP levels were determined by a ligand blot technique (Gallacher et al. 1992) modified from that developed by Hossenlopp, Seurin, Segovia-Qulnson, Hardouin & Binoux (1986). Plasma (2 μl) was diluted to 20 μl with 0.01 M PBS pH 7.4 and then mixed 1:1 with 20 μl non-reducing sample buffer (10% glycerol, 3% sodium dodecyl sulphate (SDS), 12.5% 0.05 M Tris, pH 6.8). After boiling for 3 min the samples were loaded onto a 12% polyacrylamide gel which was run at 30 mA with a 0.025 M Tris-glycine buffer (pH 8.3) containing 0.1% SDS using a Biorad Protean II electrophoresis system (Biorad, Auckland, NZ). The proteins were then transferred to nitrocellulose membranes using a constant 100 mA current overnight and 0.015 M Tris-glycine buffer (pH 8.3) containing 5% methanol in a Biorad Transblot apparatus. The nitrocellulose was air-dried and washed sequentially in 0.05 M Tris buffered saline (pH 7.4) containing 3% Tween 20 (30 min), Tris buffered saline containing 1% BSA (2 h) and Tris buffered saline containing 0.1% Tween 20 (15 min). The blot was incubated with 125I labelled rhIGF-II (150,000 cpm/ml) overnight at 4°C and then washed twice in Tris buffered saline. The nitrocellulose filters were air-dried and exposed to Kodak X-OMAT AR diagnostic film in Amersham Hyperscreen cassettes with intensifier screens to detect the IGFBP bands. The labelled IGF-II binding observed was fully displaceable in a dose-dependent manner by unlabelled IGF-I or IGF-II. Approximate molecular sizes of the ligand blot bands were obtained by calibration with Amersham 14C-labelled molecular weight markers. The bands were identified with reference to published ovine IGFBP data (Walton, Grant, Owens, Wallace & Ballard 1990). The radioactivity of each IGFBP band cut from the nitrocellulose sheet was measured and the plasma levels were expressed relative to the values obtained for each IGFBP during the control period. Samples were measured in triplicate and responses from each animal were measured on the same gel. The within and between gel variation in the measurement of samples was 14% and 19% respectively.
(vi) Circulating Type 2 IGF Receptor.

Plasma levels of the circulating type 2 receptor were measured by Dr W. Kiess (University of Munich, Germany) using a Western immuno-blot technique (Kiess et al. 1987). IGF-II receptor antibody (#3637) was raised in a rabbit against purified IGF-II receptor from rat chondrosarcoma cells, and gammaglobulins from the resultant antiserum were purified on a protein A affinity column. Plasma samples were chromatographed on a Sephadex G-200 column (Pharmacia, Uppsala, Sweden) with 0.05 M NH₄HCO₃ (pH 8.0) as the eluting buffer. The protein profile was determined by monitoring absorbance at 280 nm. The void volume fractions were collected and subjected to 6% SDS-polyacrylamide gel electrophoresis and the electrophoresed proteins transferred onto nitrocellulose membranes. The nitrocellulose was then incubated with the type 2 receptor antibody (#3637) for 3 h and then with ¹²⁵I-protein A for 1 h. The nitrocellulose membrane was then exposed to autoradiographic film with intensifier screens at -70°C. The amount of IGF-II receptor in each plasma sample was quantified by densitometry and expressed relative to control concentrations.

(vii) PL Assay.

Recombinant oPL (Genentech, South San Francisco, USA) was iodinated by a modification of the lactoperoxidase method (Gluckman et al. 1979) and the ¹²⁵I-oPL was then purified by exclusion gel chromatography on a Sephadex G-100 column (90 X 1.1 cm, Pharmacia, Uppsala, Sweden) using 0.05 M barbitone pH 8.6 as the eluting buffer. The specific activity of ¹²⁵I-labelled roPL was 40 μCi/μg. The RIA buffer was the same as that used in the IGF-I RIA. The incubation volume was 500 μl and a rabbit anti-oPL antiserum (RASPL#1) was used at a final concentration of 1:150,000. This antiserum had been raised to a purified preparation of oPL (Gluckman & Barry 1988). The assay consisted of a 24 h pre-incubation period at room temperature where 300 μl of the antibody mixture was added to tubes containing 100 μl of standard or sample diluted in assay buffer. The tracer, diluted to 100 μl/tube (25,000 cpm) was then added and the incubation continued for another 24 h at 4°C. Free and bound tracer were separated by the addition to each tube of 1 ml of a pre-precipitated second antibody mixture containing 0.01 M PBS, 1% (v/v) sheep anti-
rabbit gammaglobulin, 0.05% (v/v) normal rabbit serum and 5% (w/v) PEG 6000. The tubes were centrifuged and counted under the same conditions as in the previously described RIA methods. The minimal detectable dose was 0.05 ng/tube, half displacement was achieved at 0.6 ng/tube, the intra-assay coefficient of variation was 5.1% and the interassay coefficient of variation was 9.7%. Plasma concentrations are expressed in terms of recombinant oPL (batch # 11380/77, Genentech, San Francisco, California, USA).
DATA ANALYSIS.

Maternal and fetal plasma PL concentrations show marked inter-animal variation as has been reported elsewhere (Taylor et al. 1980; Brinsmead et al. 1981; Butler et al. 1987). Plasma IGF and insulin concentrations also exhibit a lesser degree of inter-animal variation. For this reason all endocrine and metabolite data were normalised relative to the circulating concentrations observed during control periods in each individual animal. The normalised data were used for graphical presentation in Chapters 3, 4, and 5 and for some statistical methods. Normalised data were used rather than percentage change for all endocrine and metabolite data except plasma IGFBP and type 2 receptor data because this provides an appreciation of the absolute magnitude of the change. For example, if a hormone normally circulates at a plasma concentration of 5 nM and falls to 2.5 nM that is the same percentage fall as a different hormone falling from 300 nM to 150 nM but because the absolute change is different the physiological relevance may be different. Because plasma IGFBPs were measured in terms of ability to bind radiolabelled ligand after electrophoresis and these measurements were not calibrated against known quantities of IGFBP treated in the same manner the results obtained cannot be considered to be representative of true plasma concentrations. IGFBP data from the various manipulations are presented in tabular form as a proportion of the binding found in ligand blots of plasma from paired control observations and as percentage change from control for graphical presentation. In text IGFBP data derived from ligand blot analysis will be expressed as a relative level rather than as an absolute unit of concentration. Because a specific antibody is used to detect Western blots of type 2 receptor, comparison between control and test observations will give relative plasma concentrations. As there were no standards available for analysis of plasma type 2 receptor absolute units of concentration cannot be given. Thus, plasma type 2 receptor data from test samples are presented as percentage change from control in graph format and as a proportion of control plasma concentration in tabular format. Absolute data for all other endocrine measurements are presented in tabular form. Data are expressed as mean ± SD.
For statistical comparison between single absolute values paired T-tests were used (e.g. control value versus starvation value). Grouped data were analysed for difference between sequential treatments by factorial analysis of variance (e.g. comparison between multiple values from a saline infusion period versus those from a hormone infusion period). Comparison between groups of animals used normalised data and was performed by 2 way analysis of variance with repeated measures (e.g. amino acid infusion group versus glucose infusion group). For differences between groups at specific time points factorial analysis of variance was used. The Sheffé F test was used to correct for multiple comparisons where made. Significant differences were accepted at the 5 % level.
CHAPTER 3. NUTRITIONAL STATUS: INFLUENCES ON PLASMA IGF-I, INSULIN AND PL CONCENTRATIONS.

INTRODUCTION.

The influence of nutritional status on circulating concentrations of IGF-I postnatally is well recognised and has been reviewed in Chapter 1. In an earlier study we investigated the effect of 72 h maternal starvation and refeeding on fetal plasma IGF-I concentrations (Bassett et al. 1990c). In that study maternal and fetal plasma IGF-I concentrations decreased during starvation but returned to pre-starved values after both maternal glucose infusion in the last 4 h of starvation and a subsequent 48 h period of maternal refeeding. In later experiments it became apparent that nutritional status of the ewe prior to starvation had an influence on plasma IGF-I responses to starvation. The earlier protocol was therefore modified to include two groups of ewes that were placed on specified levels of feed intake prior to the start of the experiment.

In an unrelated series of pilot experiments we observed a divergent fetal plasma IGF-I response to fetal infusion of IGF-I that was dependant on the prior nutritional status of individual ewes. The fall in fetal plasma IGF-I concentrations on maternal starvation (Bassett et al. 1990c) and the possible influence of prior nutritional status raises some questions about the influence of maternal nutrition on the IGF carrying capacity of the fetal circulation. To further determine the effect of maternal nutrition on fetal plasma IGF-I carrying capacity we have infused a low dose of IGF-I into fetuses whose mothers had either been maintained on a low or high level of feed intake prior to the study.

The influence of prior maternal nutritional status on fetal plasma PL responses to maternal starvation and refeeding was also studied using our earlier protocol (Bassett et al. 1990c). The literature concerning the influence of nutrition on circulating concentrations of PL is contradictory with some studies reporting no influence of starvation on maternal PL concentrations (Butler et al. 1987), while others report that longer term nutritional restriction leads to an increase in maternal PL concentrations (Brinsmead et al. 1981). Fetal plasma PL
concentrations have been reported to increase on 72 h maternal starvation (Freemark et al. 1989), but the potentially confounding influence of prior nutritional status had not been investigated.

In designing protocols natural variation in the circulating concentrations of the hormones studied have to be taken into consideration. Variation in the circulating concentrations of glucose, insulin and the IGFs may occur with changes in the diurnal nutritional status of the mother. Therefore sampling was performed at set times during the day, usually before morning and afternoon feeding. If the experiment required frequent sampling throughout a given day, as was the case with the IGF-I infusion experiment, the daily feed ration was divided into smaller portions and fed to the ewes more frequently to minimise fluctuation in nutrient supply. The split feeding was started the day after surgery and continued for at least a week before experiments were started. Because of the complexities of ruminant nutrition this feeding strategy should be used for as long as possible prior to commencement of the experiment (Mellor 1987). Using our feeding strategy as described above there was little diurnal variation in the plasma concentration of any of the hormones measured, apart from maternal PL which fluctuates with no apparent rhythm (Butler et al. 1987).

MATERIALS AND METHODS.


The protocol was designed to provide paired, within animal comparisons between different phases of the experiment. This type of has the disadvantage of not including concurrent controls but has the advantage of limiting the number of preparations needed and is not so adversely affected by inter-animal variation. The protocol consisted of a 48 h control period, a 72 h maternal starvation terminated by an intravenous infusion of 50 g glucose to the mother over 4 h and then 48 h maternal ad libitum refeeding. For at least a week before, during the control period and during the refeeding five ewes were maintained on a higher level of feed intake (1200 to 1600 g sheep nuts/day) and five others placed on a lower level of feed intake (500 g sheep nuts/day). For a number of reasons it was not practical to extend the period of
conditioning to these feeding regimes although this would have been more ideal. Paired maternal and fetal blood samples were taken twice daily and an extra sample was taken following the completion of the maternal glucose infusion. Doubling of the fetal blood glucose concentration in the fetal sheep over 24 h is associated with increased fetal oxygen consumption and pCO₂ but no large change in fetal pH (Philipps, Porte, Stabinski, Rosenkrantz & Raye 1984). Because of the large amount of glucose being infused care had to be taken that fetal hyperglycaemia did not cause fetal pO₂ or pH to fall too far. If fetal pO₂ fell below 16 mm Hg or pH below 7.3 we planned to slow the infusion rate, but this was never necessary.

(ii) Fetal Infusion of IGF-I Protocol.

Ewes were maintained on the previously described higher level of feed intake (n=3) or lower level of feed intake (n=3) for a week before the fetal infusions were started. Saline was infused via the fetal tarsal vein for 24 h and then IGF-I (3 μg/kg/h, Ciba, Basel, Switzerland) was infused for 24 h. Paired maternal and fetal samples were taken 1 h (-1) before the start of each infusion, at time 0, and then at +1, +2, +3, +4, +5, +19, +20, +21, +22, +23 and +24 h of infusion. On the three days following the completion of the IGF-I infusion, samples were taken at times that corresponded to samples taken at -1 and 0 h.
RESULTS.

(i) Metabolites, blood gases and pH.

Blood glucose and lactate measurements were obtained for only 4 animals studied because glucose measurements obtained during early experiments were found to be unreliable. In later experiments when reliable measurements were available maternal and fetal blood glucose concentrations fell by 50% during 72 h starvation (maternal 2.2 ± 0.3 to 1.1 ± 0.2 mM, \( p = 0.01 \), fetal 0.8 ± 0.2 to 0.4 ± 0.1 mM, \( p = 0.01 \)). Maternal and fetal blood lactate concentrations were unaffected by maternal starvation (maternal 0.5 ± 0.2 to 0.4 ± 0.3 mM, fetal 1.5 ± 0.5 to 1.7 ± 1.1 mM). During maternal glucose infusion both maternal and fetal glucose concentrations increased to 4 times the values observed during the control period (maternal 9.0 ± 2.3 mM and fetal 3.9 ± 1.0 mM, both \( p < 0.05 \)). Maternal and fetal blood lactate concentrations did not change during glucose infusion (maternal 0.4 ± 0.2 mM, fetal 2.3 ± 0.6 mM, \( p = 0.2 \)). Fetal \( \text{pO}_2 \) and pH fell slightly during the glucose infusion (\( \text{pO}_2 \) 26.3 ± 3.6 to 25.2 ± 2.9 mm Hg, pH 7.35 ± 0.02 to 7.31 ± 0.01, \( p < 0.01 \)).

(ii) IGF-I.


The level of feed intake by ewes prior to the experiment had no significant influence on her control plasma IGF-I concentrations (Table 3a). In both groups maternal and fetal plasma IGF-I concentrations fell in response to 72 h starvation, but IGF-I concentrations in ewes on the high level of feed intake fell faster, and relatively further, than in ewes on the low level of feed intake (Fig 3a and Table 3a). There were no significant differences in fetal plasma IGF-I concentrations between the groups.

Fetal Infusion of IGF-I.

Fetal plasma IGF-I concentrations were not different between the two groups. During the fetal infusion of IGF-I plasma IGF-I concentrations from fetuses whose mothers had been on a low level of feed intake tended to increase (not significant, \( n = 3 \)) in response to the infusion whereas there was no change in those from a high level of feed intake (Fig 3b and Table 3b).

Figure 3a. Values are means ± SD, calculated for the change from mean baseline IGF-I concentration for each animal. Starvation began at 0 h. The solid bar indicates i.v. glucose infusion to the mother. Filled circles, low level of feed intake (n = 5). Open circles, high level of feed intake (n = 5). *, Significant differences between the nutritional groups (p < 0.05).
Table 3a. Maternal and Fetal Plasma Hormone Concentrations in Response to Maternal Starvation, Infusion of Glucose and Refeeding.

<table>
<thead>
<tr>
<th></th>
<th>Maternal</th>
<th>Fetal</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Low Feed</td>
<td>High Feed</td>
</tr>
<tr>
<td><strong>IGF-I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>22.4 ± 11.4</td>
<td>33.9 ± 7.7</td>
</tr>
<tr>
<td>72 h Starved</td>
<td>11.6 ± 5.6</td>
<td>15.6 ± 5.9*</td>
</tr>
<tr>
<td>Post Infusion</td>
<td>9.7 ± 5.3</td>
<td>13.6 ± 3.3</td>
</tr>
<tr>
<td>Refeeding</td>
<td>20.2 ± 11.8</td>
<td>20.9 ± 3.5</td>
</tr>
<tr>
<td><strong>Insulin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.3 ± 0.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>72 h Starved</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Post Infusion</td>
<td>0.7 ± 0.3</td>
<td>0.9 ± 0.7</td>
</tr>
<tr>
<td>Refeeding</td>
<td>0.3 ± 0.2*</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td><strong>oPL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>16.3 ± 6.8</td>
<td>13.1 ± 3.9</td>
</tr>
<tr>
<td>72 h Starved</td>
<td>18.6 ± 7.5</td>
<td>19.1 ± 8.0</td>
</tr>
<tr>
<td>Post Infusion</td>
<td>16.0 ± 6.9</td>
<td>21.7 ± 5.9</td>
</tr>
<tr>
<td>Refeeding</td>
<td>20.5 ± 9.6</td>
<td>15.5 ± 4.6</td>
</tr>
</tbody>
</table>

Plasma concentrations (nM), are means ± SD for 5 animals in each group. Control, last measurement before starvation; 72 h Starved, value after 72 h of starvation; Post Infusion, value after 4 h of glucose infusion; Refeeding, value after 40 h of ad libitum refeeding. * Significantly different from earlier value in same group (p < 0.05).

Table 3b. Maternal and Fetal Plasma IGF-I Responses to Fetal Infusion of IGF-I.

<table>
<thead>
<tr>
<th></th>
<th>Maternal</th>
<th>Fetal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low Feed</td>
<td>High Feed</td>
</tr>
<tr>
<td><strong>Saline Infusion</strong></td>
<td>11.8 ± 2.5</td>
<td>31.8 ± 3.7</td>
</tr>
<tr>
<td><strong>IGF-I Infusion</strong></td>
<td>12.8 ± 1.9</td>
<td>33.2 ± 5.8</td>
</tr>
<tr>
<td><strong>Post Infusion</strong></td>
<td>14.8 ± 2.3</td>
<td>29.8 ± 5.0</td>
</tr>
</tbody>
</table>

Plasma concentrations (nM), are means ± SD of 10 values from each of the 3 study periods (n = 3 animals per feeding group).
Fetal Plasma IGF-I Responses to Fetal Infusion of IGF-I.

Figure 3b. Values are mean ± SD, calculated for the change from the mean saline infusion plasma IGF-I concentration for each animal. The solid bar indicates i.v. IGF-I infusion to fetuses. Filled circles, low level of feed intake (n = 3). Open circles, high level of feed intake (n = 3).

(iii) Insulin.

Maternal and fetal plasma insulin concentrations fell during starvation in both groups, but fell faster and relatively further in animals from the high level of feed intake than in animals from the low level of feed intake (Fig. 3c and Table 3a).

Plasma insulin concentrations were not measured in samples from the IGF-I infusion experiment.
Maternal and Fetal Plasma Insulin Responses to Maternal Starvation, Infusion of Glucose and Refeeding.

Figure 3c. Values are means ± SD, calculated for the change from mean baseline plasma insulin concentration for each animal. Starvation began at 0 h. The solid bar indicates i.v. glucose infusion to the mother. Filled circles, low level of feed intake (n = 5). Open circles, high level of feed intake (n = 5). *, Significant differences between the nutritional groups (p < 0.05).
Maternal and Fetal Plasma oPL Responses to Maternal Starvation, Infusion of Glucose and Refeeding.

**Figure 3d.** Values are means ± SD, calculated for the change from mean baseline plasma oPL concentration for each animal. Starvation began at 0 h. The solid bar indicates i.v. glucose infusion to the mother. Filled circles, low level of feed intake (n = 5). Open circles, high level of feed intake (n = 5). * Significant differences between the nutritional groups (p < 0.05).

(iv) PL.

There was high inter-animal variation in plasma oPL concentration in mothers and fetuses. It is possible that this high inter-animal variation prevented significant differences in basal maternal and fetal plasma oPL concentrations being observed although there was a tendency for maternal and fetal plasma oPL concentrations to be higher
in the low feed intake group (Table 3a). During maternal starvation, plasma oPL concentrations from fetuses whose mothers had a previously high level of feed intake were significantly higher (Fig. 3d) than those whose mothers had a low level of feed intake. Maternal oPL concentrations followed similar trends but the difference between the groups did not reach statistical significance (Fig. 3d).

Infusion of 50 g glucose over 4 h into the fasted ewe caused a 2-3 fold increase in fetal plasma PL concentrations (Fig. 3d and Table 3a). Maternal plasma PL concentrations were unaffected by the infusion. During 48 h of maternal *ad libitum* refeeding both fetal and maternal plasma PL concentrations returned to near control values.

Plasma oPL concentrations were not measured in samples from the IGF-I infusion studies.
DISCUSSION.

(i) IGF-I.

Many studies have reported the relationship between nutrition and endocrine IGF-I (Clemmons & Underwood 1991; Chapter 1). The decreases in maternal and fetal plasma IGF-I concentrations during starvation are compatible with previous reports. Maternal plasma IGF-I concentrations fell faster on starvation in the high feed intake group than in the low feed intake group but this difference was not apparent in fetal data. A longer term of conditioning to either nutritional plane may have lead to a greater separation in plasma IGF-I concentrations between the two nutritional groups. The more marked effect of prior nutritional status on maternal plasma IGF-I responses to starvation may lend support the hypothesis that fetal plasma IGF-I is regulated independently of that of the mother. This will be discussed further in Chapter 4.

Infusion of glucose to the ewe had little effect on maternal or fetal plasma IGF-I concentrations despite the fact that maternal and fetal glucose concentrations were markedly elevated. In our previous studies maternal and fetal plasma IGF-I concentrations had returned to near control values during 48 h maternal refeeding (Bassett et al. 1990c). This may suggest that more than 4 h of glucose elevation is required to restore fetal plasma IGF-I concentrations. However, it was also possible that glucose alone was not the major regulator of fetal plasma IGF-I concentration and that other nutrients were involved. It was also not clear from these studies to what extent fetal plasma IGF-I concentration was regulated by fetal metabolic and endocrine factors independently of maternal factors.

Limited data from the fetal IGF-I infusion experiment suggest that prior maternal nutritional status influences fetal plasma IGF-I response. The fetuses whose mothers had been on a high level of feed intake exhibited no increase in fetal plasma IGF-I concentration on IGF-I infusion, whereas in fetuses whose mothers had been on a low level of feed intake fetal plasma IGF-I concentrations increased. It should be noted that on fetal infusion of IGF-I, absolute plasma IGF-I
concentrations in the low feed intake group increased to the same concentration as the high feed intake group, but no higher. Nutrition plays an important role in the regulation of circulating concentrations of both maternal and fetal IGFBPs (Clemmons & Underwood 1991). Fetal plasma IGFBP-1 and BP-2 levels, as assessed by ligand blot, increase on maternal starvation (Gallaher et al. 1992) and so may have been more abundant in the blood of fetuses from the low nutrition group. These two IGFBPs bind over 40% of fetal plasma IGF-I in normal circumstances (Gallaher et al. 1992). From the same studies we reported that total fetal plasma IGF-I binding capacity increased after 72 h of maternal fasting. Therefore when IGF-I was infused into fetuses from the low feed intake group a greater proportion of the exogenous IGF-I may have been retained in the circulation by binding to elevated plasma concentrations of IGFBP-1 and BP-2. In contrast, fetal plasma from well fed mothers may have had relatively lower IGFBP-1, BP-2 concentrations and total IGF-I binding capacity so the exogenous IGF-I may be cleared faster from the circulation. The effects of maternal starvation on fetal plasma IGFBP levels as measured by ligand blot are characterised in Chapter 4.

(ii) PL

The fetal plasma oPL response to starvation depended on the prior level of maternal feed intake (Oliver, Harding, Breier, Evans & Gluckman 1992). Fetal plasma oPL concentrations increase during 72 h of maternal starvation (Freemark et al. 1989) and in our study a similar increase in plasma oPL concentration was observed in fetuses from previously well fed ewes. However, plasma oPL concentrations in fetuses whose mothers had previously been on a low level of feed intake did not increase in response to starvation (Oliver et al. 1992). The postulated role for PL in the mother is to protect fetal substrates through preferential induction of lipolysis (Thordarson et al. 1987). The increase in plasma oPL concentrations seen in fetuses from previously well fed mothers may suggest that the hormone is functioning in a similar manner in the fetus. In fetuses from the poorly fed ewes it is possible that a similar response did not occur, or was delayed. Alternatively, the number of the putative oPL receptors may differ in fetuses in either nutritional group. It has been reported that fetal oPL receptors are reduced in number after 72 h maternal fasting.
in sheep (Freemark et al. 1989). In our study, following a similar period of starvation, a reduction in the number of fetal oPL receptors may be associated with an increase in the plasma oPL concentrations in fetuses from previously well fed mothers. If in fetuses from poorly fed mothers, the PL receptors had been down regulated prior to the period of starvation, compensatory increases in fetal plasma oPL concentration would be unlikely.

In the ewe, it has been reported that plasma oPL concentrations vary considerably in individual animals over time (Butler et al. 1987; Taylor et al. 1980), apparently showing no clear rhythm. This variation may have the effect of masking most changes in hormone concentrations that may occur in response to experimental manipulations. However during starvation, ewes from the high feed intake group tended to have higher plasma oPL concentrations than those from the low feed intake group which was consistent with the fetal observations. PL has recently been shown to stimulate appetite in lambs (Breier & Gluckman, unpublished observations). The tendency for basal plasma oPL concentrations to be higher in ewes from the lower feed intake group and for plasma oPL concentrations to increase upon starvation in the higher nutrition group may be compatible with PL having a role in stimulating appetite. Longer term manipulation of pre-starvation nutrition may have caused a significant separation in maternal plasma oPL concentrations between the two groups and therefore provided better support for the suggestion that PL has a role in appetite.

The acute rise in fetal plasma oPL concentration seen during maternal infusion of glucose (Oliver et al. 1992) was not expected. Indeed the opposite effect might have been expected given that PL may have a substrate releasing action postnatally (Thordarson et al. 1987). However, in human term placental explants physiological concentrations of glucose had a releasing action on PL secretion (Belleville, Lasbennes, Nabet & Paysant 1979). In vitro studies also demonstrate that PL promotes glycogen synthesis in fetal sheep hepatocytes (Freemark & Handwerger 1986). Similarly, in human term placental explants IGF-I and insulin induce an increase in PL content in culture medium (Bhaumick, Dawson & Bala 1987). The large increases in fetal plasma
3. Nutritional Status and Fetal IGFs, Insulin and PL.

oPL and insulin concentrations seen in the current study following the glucose infusion may reflect these actions in vivo.

There was no change in maternal plasma oPL concentration during maternal infusion of glucose even though maternal plasma insulin concentrations showed a significant increase, suggesting that the functional relationship between circulating concentrations of glucose, insulin and PL differs in the adult and the fetus. Other studies in the pregnant ewe however, demonstrate that maternal plasma oPL concentrations increase following the infusion of other metabolic substrates such as amino acids (Handwerger et al. 1978; Handwerger et al. 1981) and high density lipoproteins (Grandis et al. 1989).

In summary, maternal and fetal plasma IGF-I, glucose and insulin concentrations were depressed by maternal starvation. Maternal plasma IGF-I responses, and both maternal and fetal plasma insulin responses to maternal starvation exhibited some dependence on the prior feed intake of the mother. Prior nutritional status of the ewe also influenced fetal plasma oPL responses to maternal starvation. Maternal infusion of glucose at the end of starvation had little effect on fetal plasma IGF-I concentration but caused marked increases in maternal and fetal plasma insulin concentrations, and fetal plasma PL concentration. Maternal plasma PL concentration was not affected by glucose infusion. Lastly, data from the nutritional manipulation studies highlight the importance of determining pre-existing maternal nutritional status in any study involving the relationships between fetal substrate supply and the plasma concentrations of IGF-I, insulin and PL.
CHAPTER 4. EFFECTS OF FETAL INFUSION OF METABOLITES ON BLOOD METABOLITE, PLASMA IGF, IGFBP, INSULIN AND PL CONCENTRATIONS.

INTRODUCTION.
Maternal starvation caused a fall in fetal plasma IGF-I concentrations and 48 h maternal refeeding with glucose priming returned them to near control values (Chapter 3, Bassett et al. 1990). It was not clear from those experiments whether fetal plasma IGF-I concentration was directly regulated by fetal glucose or other metabolites, or whether maternal factors were responsible. To clarify this question, we infused glucose or amino acids into fetuses of starved ewes and studied the effects on fetal plasma IGF-I and IGF-II concentrations. Since a pharmacological dose of glucose to the ewe at the end of starvation caused a marked increase in fetal plasma oPL concentration (Oliver et al. 1992; Chapter 3), we also determined whether physiological doses of glucose or amino acids caused a similar oPL response.

Prior maternal nutritional status may influence the fetal plasma IGF-I responses to fetal infusion of IGF-I as suggested in Chapter 3. One possible reason for this observation is that the plasma IGF-I clearance may have been different between the two groups of fetuses. Fetal plasma IGFBP concentrations may have been different between the two groups since IGFBPs have been reported to be under strong nutritional influence (Clemmons & Underwood 1991; Chapter 1). Differences in plasma IGFBP concentrations may have influenced clearance of infused IGF-I. In order to address this possibility the effect of maternal starvation and fetal glucose replacement on maternal and fetal plasma IGFBP levels will be described.

MATERIALS AND METHODS.

(i) Maternal Starvation/Fetal Infusion of Glucose or Amino Acid Protocol.
A design that used paired, within animal comparison was once again used for these experiments. The experimental protocol consisted
of a two day control period followed by three days of maternal starvation. After 48 h of maternal starvation the fetuses were infused intravenously for 24 h with glucose (n = 6); 1 ml bolus then 2 ml/h 50% glucose solution, or amino acids (n = 5); 1 ml bolus then 3.5 ml/h 10% amino acid solution (Synthamin 17, Baxter Pharmaceuticals Ltd, NSW, Australia, Table 4a). The observations obtained from day 3 of the maternal/starvation experiments (Chapter 3) provide convenient comparison for observations during fetal infusion of glucose or amino acids. Care was taken to shield the amino acid infusate from light to prevent amino acid degradation. The infusion rates were equivalent to estimated fetal consumption

**Table 4a: Composition of Amino Acid Mixture Synthamin 17**

Data supplied by manufacturer.

<table>
<thead>
<tr>
<th>Essential Amino Acids</th>
<th>mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Leucine</td>
<td>7.3</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>6.0</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>5.8</td>
</tr>
<tr>
<td>L-Valine</td>
<td>5.8</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>5.6</td>
</tr>
<tr>
<td>L-Histadine</td>
<td>4.8</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>4.2</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>4.0</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>1.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nonessential Amino Acids</th>
<th>mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>20.7</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>11.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.3</td>
</tr>
<tr>
<td>L-Serine</td>
<td>5.0</td>
</tr>
<tr>
<td>L-Proline</td>
<td>6.8</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>0.4</td>
</tr>
</tbody>
</table>

rates (Battaglia & Meschia 1978). Fetal blood gases, pH and glucose and lactate concentrations were measured frequently during the first 4 h of the infusions to ensure the fetuses were not becoming acidotic or hypoxic (pH below 7.3 or pO2 below 16 mmHg), but this did not occur. Frequent measurement of fetal blood glucose concentration during glucose infusion allowed adjustment of the infusion rate to maintain fetal blood glucose concentrations near control values. After 3 to 4 h infusion of 50% glucose solution at 2 ml/h fetal blood glucose
concentrations usually began to fall, and it was necessary to supplement fetal glucose supply with an additional 10% glucose infusion (usually at 2 to 3 ml/h). Similar adjustments were not made during amino acid infusion because we had no method for rapid measurement of fetal blood amino acid concentrations. Paired maternal and fetal blood samples were taken daily in the morning before normal feeding time and in the afternoon 8 h later. On the day of the fetal infusion additional samples were taken 4 h before and immediately before the start of the infusion (0 h), and then at +2, +4, +8 and +24 h of the infusion. The ewes were refed ad libitum for 48 h after the completion of the infusions.
RESULTS.
(i) Metabolites.

Starvation.

Maternal and fetal blood glucose concentrations fell rapidly during maternal starvation (Fig. 4a and table 4b). Maternal blood lactate concentration did not change during starvation, while the fall in fetal blood lactate concentration did not reach significance (Fig. 4b and Table 4b). Maternal and fetal blood AN concentration did not change significantly during starvation (Fig. 4c and Table 4b). Apart from glucose the changes in all blood metabolite concentrations were small and did not reach statistical significance.

Glucose infusion.

Fetal blood glucose concentration was returned to control values by glucose infusion, and remained there during maternal refeeding (Fig and Table 4b). Maternal blood glucose concentration remained depressed during the fetal infusion. Fetal blood lactate concentration was unchanged during glucose infusion, but increased on maternal refeeding (Fig. 4b and Table 4b). An increase in maternal blood lactate concentration in the first 24 h of maternal refeeding did not reach significance. Blood AN concentrations were only measured in one animal from these studies. In the one animal studied, fetal blood AN concentrations fell during glucose infusion and maternal refeeding (Table 4b). Maternal blood AN concentrations remained depressed during glucose infusion and maternal refeeding in that animal.

Amino Acid Infusion.

Fetal blood glucose concentrations remained depressed during fetal infusion of amino acids but increased on maternal refeeding (Fig 4a and Table 4b). Maternal blood glucose concentration continued to fall during amino acid infusion and after 8 h was lower than in the fetal glucose infusion group. Fetal and maternal blood lactate responses to amino acid infusion were similar to the glucose infusion group (Fig. 4b and Table 4b). Fetal blood AN concentrations did not increase significantly during amino acid infusion (Fig. 4c and Table 4b). Maternal blood AN concentrations remained constant during the fetal infusion and during maternal refeeding.
Table 4b. Blood Metabolite Concentrations During Maternal Starvation, Infusion of Glucose or Amino Acids to the Fetus and Maternal Refeeding.

<table>
<thead>
<tr>
<th></th>
<th>Glucose (mM)</th>
<th>AN (mM)</th>
<th>Lactate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FETAL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose Infusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.8 ± 0.2</td>
<td>7.4†</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>48 h Starved</td>
<td>0.5 ± 0.1*</td>
<td>6.0†</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>24 h Infusion</td>
<td>0.8 ± 0.2*</td>
<td>5.3†</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>48 h Refeeding</td>
<td>0.9 ± 0.3</td>
<td>4.8†</td>
<td>1.1 ± 0.2*</td>
</tr>
<tr>
<td>Amino Acid Infusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.9 ± 0.2</td>
<td>8.2 ± 0.3</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>48 h Starved</td>
<td>0.6 ± 0.2*</td>
<td>8.2 ± 0.7</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>24 h Infusion</td>
<td>0.5 ± 0.2</td>
<td>8.9 ± 0.4</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>48 h Refeeding</td>
<td>0.9 ± 0.5</td>
<td>7.3 ± 0.8*</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td><strong>MATERNAL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose Infusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.4 ± 0.3</td>
<td>4.5†</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>48 h Starved</td>
<td>1.4 ± 0.5*</td>
<td>3.4†</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>24 h Infusion</td>
<td>1.4 ± 0.3</td>
<td>3.7†</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>48 h Refeeding</td>
<td>2.5 ± 0.4*</td>
<td>2.8†</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Amino Acid Infusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.5 ± 0.4</td>
<td>4.3 ± 0.7</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>48 h Starved</td>
<td>1.4 ± 0.4*</td>
<td>3.6 ± 0.4</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>24 h Infusion</td>
<td>1.2 ± 0.4</td>
<td>3.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>48 h Refeeding</td>
<td>2.5 ± 1.3</td>
<td>3.6 ± 0.3</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

Blood concentrations (mM) are means ± SD (n = 6 glucose infusion group and n = 5 amino acid infusion group). Control, last measurement before starvation; 48 h Starved, value after 48 h starvation; 24 h Infusion, value after 24 h infusion to the fetus; 48 h Refeeding, value after 48 h maternal refeeding. *, Significantly different from previous value (p < 0.05). AN; amino nitrogen. †, Data only available from 1 animal.
Maternal and Fetal Blood Glucose Responses to Starvation.

Fetal Infusion of Glucose or Amino Acids and Maternal Refeeding.

Figure 4a. Values are means ± SD, calculated for the change from mean baseline blood glucose concentration for each animal. Starvation began at 0 h. The solid bar indicates i.v. glucose or amino acid infusion to the fetus. Filled circles, amino acid infusion (n = 5). Open circles, glucose infusion (n = 6). *, Significant differences between the infusion groups (p < 0.05).
Maternal and Fetal Blood Lactate Responses to Starvation.

Fetal Infusion of Glucose or Amino Acids and Maternal Refeeding.

**Figure 4b.** Values are means ± SD, calculated for the change from mean baseline blood lactate concentration for each animal. Starvation began at 0 h. The *solid bar* indicates i.v. glucose or amino acid infusion to the fetus. *Filled circles*, amino acid infusion (n = 5). *Open circles*, glucose infusion (n = 6).
Maternal and Fetal Blood AN Responses to Starvation, Fetal Infusion of Glucose or Amino Acids and Maternal Refeeding.

Figure 4c. Values are means ± SD, calculated for the change from mean baseline blood AN concentration for each animal. Starvation began at 0 h. The solid bar indicates i.v. amino acid infusion to the fetus (n = 5).
Maternal and Fetal Plasma IGF-I Responses to Starvation.
Fetal Infusion of Glucose or Amino Acids and Maternal Refeeding.

![Graph showing change in plasma IGF-I](image)

Figure 4d. Values are means ± SD, calculated for the change from mean baseline plasma IGF-I concentration for each animal. Starvation began at 0 h. The solid bar indicates i.v. glucose or amino acid infusion to the fetus. Filled circles, amino acid infusion (n = 5). Open circles, glucose infusion (n = 6). *~, Significant differences between the infusion groups (p < 0.05).

(ii) IGF-I.
Maternal and fetal plasma IGF-I concentrations fell during maternal starvation (Fig. 4d and Table 4c). Infusion of glucose, but not amino acids, to fetuses caused fetal plasma IGF-I concentrations to
increase to near control values. Fetal plasma IGF-I concentrations were near control values after 48 h maternal *ad libitum* refeeding while maternal plasma IGF-I concentrations were still lower than control values.

(iii) IGF-II

Fetal plasma IGF-II concentration fell while maternal plasma IGF-II concentration rose slightly during maternal starvation (Fig 4e and Table 4c). Infusion of glucose to fetuses returned fetal plasma IGF-II concentrations to control values. Maternal plasma IGF-II concentration was unaffected by the fetal glucose infusion. Following 48 h maternal *ad libitum* refeeding both maternal and fetal plasma IGF-II concentrations were near control values. Plasma IGF-II concentrations were not measured in the amino acid infusion group.

**Maternal and Fetal Plasma IGF-II Responses to Starvation.**

**Fetal Infusion of Glucose and Maternal Refeeding.**

![Graph](image)

*Figure 4e. Values are means ± SD, calculated for the change from mean baseline plasma IGF-II concentration for each animal (n=6). Filled bars, maternal responses. Open Bars, fetal responses. Starved, value after 48 h starvation; Infused, value after 24 h infusion to fetus; Refed, value after 48 h maternal refeeding. *, significantly different from previous value (p < 0.05).*
### Table 4c. Maternal and Fetal Plasma Hormone Responses to Maternal Starvation, Infusion of Glucose or Amino Acids to the Fetus and Maternal Refeeding.

<table>
<thead>
<tr>
<th></th>
<th>Glucose Infusion</th>
<th>Amino Acid Infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FETAL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IGF-I (nM)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>13.5 ± 3.6</td>
<td>10.2 ± 1.8</td>
</tr>
<tr>
<td>48 h Starved</td>
<td>7.9 ± 3.1*</td>
<td>5.1 ± 1.6*</td>
</tr>
<tr>
<td>24 h Infusion</td>
<td>11.4 ± 2.9*</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>48 h Refeeding</td>
<td>13.1 ± 4.2</td>
<td>9.2 ± 3.8*</td>
</tr>
<tr>
<td><strong>IGF-II (nM)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>151.9 ± 20.5</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>48 h Starved</td>
<td>111.5 ± 8.7*</td>
<td>0.1 ± 0.1*</td>
</tr>
<tr>
<td>24 h Infusion</td>
<td>149.7 ± 15.9*</td>
<td>0.2 ± 0.1*</td>
</tr>
<tr>
<td>48 h Refeeding</td>
<td>160.7 ± 16.4</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td><strong>Insulin (nM)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>48 h Starved</td>
<td>0.1 ± 0.1*</td>
<td>0.1 ± 0.1*</td>
</tr>
<tr>
<td>24 h Infusion</td>
<td>0.2 ± 0.1*</td>
<td>0.2 ± 0.1*</td>
</tr>
<tr>
<td>48 h Refeeding</td>
<td>0.1 ± 0.1</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td><strong>PL (nM)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.6 ± 0.2</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>48 h Starved</td>
<td>0.6 ± 0.1</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>24 h Infusion</td>
<td>0.6 ± 0.1</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>48 h Refeeding</td>
<td>0.5 ± 0.1</td>
<td>0.8 ± 0.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Glucose Infusion</th>
<th>Amino Acid Infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MATERNAL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Glucose Infusion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>43.6 ± 11.5</td>
<td>52.8 ± 30.4</td>
</tr>
<tr>
<td>48 h Starved</td>
<td>13.0 ± 1.9*</td>
<td>11.9 ± 4.1*</td>
</tr>
<tr>
<td>24 h Infusion</td>
<td>15.4 ± 2.2</td>
<td>8.7 ± 2.7*</td>
</tr>
<tr>
<td>48 h Refeeding</td>
<td>26.3 ± 8.1*</td>
<td>14.4 ± 6.3</td>
</tr>
<tr>
<td><strong>Amino Acid Infusion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>43.6 ± 11.5</td>
<td>52.8 ± 30.4</td>
</tr>
<tr>
<td>48 h Starved</td>
<td>13.0 ± 1.9*</td>
<td>11.9 ± 4.1*</td>
</tr>
<tr>
<td>24 h Infusion</td>
<td>15.4 ± 2.2</td>
<td>8.7 ± 2.7*</td>
</tr>
<tr>
<td>48 h Refeeding</td>
<td>26.3 ± 8.1*</td>
<td>14.4 ± 6.3</td>
</tr>
</tbody>
</table>

Plasma concentrations (nM) are means ± SD (n = 6 glucose infusion group and n = 5 amino acid infusion group). Control, last measurement before starvation; 48 h Fasting, value after 48 h fasting; 24 h Infusion, value after 24 h infusion to the fetus; 48 h Refeeding, value after 48 h maternal refeeding.

* Significantly different from previous value (p < 0.05). BDL = below detectable limit (0.05 nM).

**(iv) Insulin**

Maternal and fetal plasma insulin concentrations fell during maternal starvation (Fig 4f and Table 4c). Infusion of glucose, but not amino acids, to fetuses returned fetal plasma insulin to near control concentrations.
Maternal plasma insulin concentrations were not affected by either fetal infusion and remained depressed. During 48 h maternal ad libitum refeeding both fetal and maternal plasma insulin concentrations had returned to control values.

**Maternal and Fetal Plasma Insulin Responses to Starvation.**

**Fetal Infusion of Glucose or Amino Acids and Maternal Refeeding.**

![Graph](image)

**Figure 4f.** Values are means ± SD, calculated for the change from mean baseline plasma insulin concentration for each animal. Starvation began at 0 h. The solid bar indicates i.v. glucose or amino acid infusion to the fetus. Filled circles, amino acid infusion (n = 5). Open circles, glucose infusion (n = 6). "*, Significant differences between the infusion groups (p < 0.05).
(v) IGFBPs.

Starvation.

Maternal and fetal plasma IGFBP-1 and BP-2 levels as determined by ligand blot increased during 48 h starvation (Fig. 4g and Table 4d), while maternal and fetal plasma IGFBP-3 levels decreased (Fig. 4h and Table 4d). Maternal plasma IGFBP-4 levels decreased during starvation but no change was seen in fetal plasma IGFBP-4 levels (Fig. 4h and Table 4d).


Figure 4g. Plasma IGFBP levels expressed as mean % change from control (± SD). Filled bars, maternal responses. Open Bars, fetal responses. Starved, value after 48 h starvation; Infused, value after 24 h infusion to fetus; Refed, value after 48 h maternal refeeding. *, significantly different from previous value ($p < 0.05$).
Maternal and Fetal Plasma IGFBP-3 and BP-4 Responses to Maternal Starvation, Fetal Infusion of Glucose and Maternal Refeeding.

Figure 4h. Plasma IGFBP levels expressed as mean % change from control (± SD). Filled bars, maternal responses. Open Bars, fetal responses. Starved, value after 48 h starvation; Infused, value after 24 h infusion to fetus; Refed, value after 48 h maternal refeeding. *, significantly different from previous value ($p < 0.05$).
Glucose Infusion.

Fetal plasma IGFBP-1 levels returned to control values during glucose infusion to fetuses (Fig. 4g and Table 4d), while fetal plasma IGFBP-4 levels increased (Fig. 4h and Table 4d). Fetal and maternal plasma IGFBP-2 levels were unaffected by the glucose infusion (Fig. 4g and Table 4d). IGFBP-3 levels were unchanged in fetal plasma during glucose infusion to fetuses but decreased in maternal plasma (Fig. 4h and Table 4d). Maternal plasma IGFBP-1 and IGFBP-4 levels were unaffected by the glucose infusion.

Refeeding.

During maternal ad libitum refeeding plasma levels of maternal and fetal IGFBP-1, BP-2 and BP-3 returned to near control levels as did those of fetal plasma IGFBP-4. During refeeding maternal plasma IGFBP-4 levels remained depressed. Plasma IGFBP levels were not measured in the amino acid infusion group.

Fetal Circulating Type 2 IGF Receptor Responses to Maternal Starvation, Fetal Infusion of Glucose and Maternal Refeeding.

Figure 4i. Values expressed as mean % change from control (± SE), calculated for the change from mean baseline plasma type 2 receptor concentration for each animal. Starved, value after 48 h starvation; Infused, value after 24 h infusion to fetus; Refed, value after 48 h maternal refeeding. *, significantly different from previous value (p < 0.05).
(vi) Circulating Type 2 IGF Receptor.

During maternal starvation fetal plasma circulating type 2 IGF receptor concentrations were reduced relative to control concentrations (Fig. 4i and Table 4d). Any increase in fetal plasma type 2 IGF receptor concentrations during glucose infusion to fetuses did not reach significance and there was no further change during 48 h maternal ad libitum refeeding. Maternal plasma concentrations of the circulating receptor were often below the detectable limit of the western ligand blot technique used and are not presented. Plasma type 2 receptor concentration was not measured in the amino acid infused group.

Table 4d. Maternal and Fetal Plasma IGFBP and Type 2 Receptor Responses to Maternal Starvation, Glucose Infusion to the Fetus and Maternal Refeeding.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>48 h Fasted</th>
<th>24 h Infused</th>
<th>48 h Refed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FETAL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP-1</td>
<td>1.0</td>
<td>1.32 ± 0.22*</td>
<td>0.96 ± 0.15*</td>
<td>0.99 ± 0.17</td>
</tr>
<tr>
<td>BP-2</td>
<td>1.0</td>
<td>1.20 ± 0.17*</td>
<td>1.12 ± 0.22</td>
<td>1.08 ± 0.17</td>
</tr>
<tr>
<td>BP-3</td>
<td>1.0</td>
<td>0.82 ± 0.15*</td>
<td>0.78 ± 0.15</td>
<td>0.91 ± 0.15</td>
</tr>
<tr>
<td>BP-4</td>
<td>1.0</td>
<td>0.92 ± 0.12</td>
<td>1.11 ± 0.15*</td>
<td>1.04 ± 0.15</td>
</tr>
<tr>
<td>Type 2 receptor</td>
<td>1.0</td>
<td>0.74 ± 0.07*</td>
<td>0.90 ± 0.17</td>
<td>0.89 ± 0.17</td>
</tr>
</tbody>
</table>

| **MATERNAL** |        |             |              |            |
| BP-1   | 1.0     | 1.19 ± 0.25*| 1.14 ± 0.20  | 0.97 ± 0.20 |
| BP-2   | 1.0     | 1.14 ± 0.29*| 1.17 ± 0.22  | 1.02 ± 0.20 |
| BP-3   | 1.0     | 0.78 ± 0.10*| 0.65 ± 0.15* | 0.85 ± 0.15*|
| BP-4   | 1.0     | 0.69 ± 0.10*| 0.65 ± 0.15  | 0.74 ± 0.12 |

Plasma IGFBP levels and type 2 receptor concentrations are expressed relative to control values (means ± SD, n = 6 glucose infusion group and n = 5 amino acid infusion group). Control, last measurement before starvation; 48 h Fasted, value after 48 h fasting; 24 h Infused, value after 24 h infusion to the fetus; 48 h Refed, value after 48 h maternal refeeding. *, Significantly different from previous value (p < 0.05).

(vii) PL.

Maternal starvation for 48 h, infusion of glucose or amino acids to fetuses did not cause any change in fetal or maternal PL concentrations (Table 4c).
DISCUSSION.

(i) Metabolites.

Fetal blood lactate concentrations exhibited very little change during the course of the experiment. A longer period of maternal starvation or larger amount of infused glucose may have had an influence on fetal blood glucose concentrations considering fetal glucose supply regulates lactate production (Sparks, Hay, Donds, Meschia & Battaglia 1982). Fetal blood lactate responses were the same in the amino acid infused fetuses and this may suggest that amino acids represent an alternate source of substrate for placental and fetal lactate production.

There was little change in fetal blood AN concentration during the experiment. If starvation had continued for longer an increase in fetal blood AN concentrations may have been expected as fetal and placental metabolism switched from glucose to amino acid use and fetal tissues themselves became a source of amino acids (Johnson et al. 1986; Liechty et al. 1987; Liechty et al. 1991). However, it has been shown that the concentration of most amino acids decrease in the blood of fetal sheep during starvation while those of branched chain amino acids increase (Liechty et al. 1991). Because maternal starvation may have a varied effect on individual amino acid concentrations in fetal blood, measurement of total fetal blood AN concentration may have masked significant effects.

In the one glucose-infused fetus in which it was measured fetal blood AN concentrations tended to fall during glucose infusion and maternal refeeding suggesting that fetal glucose supply may influence usage of fetal amino acids.

(ii) IGF-I.

Fetal plasma IGF-I concentrations increased from starved values during infusion of glucose to fetuses, but infusion of the amino acid mixture was without effect (Oliver, Harding, Breier, Evans & Gluckman 1993). The relative effectiveness of glucose or amino acid infusion can be by comparison with the observation that in the previous maternal starvation protocol (Chapter 3) fetal plasma IGF-I concentrations remained depressed during starvation at an equivalent
time to that of the fetal infusions. The mothers remained starved during the infusions to fetuses and maternal blood glucose, AN, plasma insulin and IGF-I concentrations remained depressed. These results suggest that fetal plasma IGF-I concentrations are regulated by fetal glucose supply and not maternal metabolic or hormonal factors.

It is not obvious from our studies why glucose rather than amino acids appears to be the important regulator of fetal plasma IGF-I concentrations since amino acids have more influence postnatally at least in other species (Isley et al. 1983; Clemmons et al. 1985). It has been previously reported that amino acids augment the fetal plasma insulin response to glucose in the fetal sheep (Fowden 1980), but the effect of restricted glucose supply on the fetal plasma insulin response to amino acids had not been reported. It may be that sufficient glucose supply has to be available before fetal plasma insulin and IGF-I responses are elicited. Another possible reason for the lack of effect of the amino acid mixture on fetal plasma insulin and IGF-I concentrations could be that the composition of amino acids may not have been appropriate and that greater concentrations of specific amino acids may have been more effective. Since fetal blood AN concentrations did not change during amino acid infusion, it is possible that a higher dose of the amino acid mixture may have had more influence on fetal plasma IGF-I concentrations. However, amino acids cross the placenta by active transport mechanisms and changes in fetal circulating amino acid concentrations are relatively small over the range of common physiological and pathological variations in fetal supply (Battaglia & Meschia 1978). Whatever mechanisms actually regulate fetal plasma IGF-I concentrations they may be more sensitive to the relatively large changes in fetal glucose rather than the relatively small changes in fetal amino acids. Teleologically, if fetal plasma IGF-I is important in the regulation of fetal growth it is more appropriate for its plasma concentrations to respond to changes in fetal blood glucose, which is not only the principle substrate for fetal metabolism (Battaglia & Meschia 1978), but also fluctuates over a wider range of concentrations in response to changes in substrate supply from the mother.

Although these studies suggest that fetal glucose is an important regulator of circulating fetal IGF-I, it is possible that insulin is also
important in this regard. In these experiments glucose, but not amino acid infusion, raised fetal plasma insulin concentrations. Thus insulin may mediate, in whole or more likely in part, the effect of glucose on fetal plasma IGF-I concentrations. This is compatible with previous findings that fetal pancreatectomy results in low fetal plasma concentrations of both insulin and IGF-I (Gluckman et al. 1987), and fetal hyperinsulinaemia leads to elevated fetal somatomedin-like bioactivity (Hill & Milner 1980; Heinze et al. 1982; Spencer et al. 1983).

Infusion of glucose to the fetus during maternal starvation may have arrested the decline in maternal plasma IGF-I concentration although this is only suggested by the data and a specific experiment would have to be designed to address the possibility. As maternal blood glucose concentrations were never less than those of the fetus it is unlikely that increased fetal glucose supply directly supplemented the maternal glucose pool as glucose is transported across the placenta by facilitated diffusion. Since the conceptus normally consumes approximately 25 % of total maternal glucose production in the sheep (Hay, Sparks, Wilkening, Battaglia & Meschia 1983), infusion of glucose to the fetus in this experiment may have reduced fetal demand for maternal glucose and thus prevented a further fall in maternal plasma IGF-I concentration, whereas in the amino acid infused group fetal glucose demand continued. Thus not only did fetal glucose supply appear to regulate fetal plasma IGF-I concentrations, but indirectly it may have a potentially important feedback influence on maternal plasma IGF-I concentrations. Alternatively, if the placenta is an additional source of IGF-I to the maternal circulation, as has been suggested previously for the fetal circulation (Iwamoto, Chernausek & Murray 1991), then an elevation in fetal glucose may affect placental IGF-I production and perhaps maternal plasma IGF-I concentrations. The observation that treatment of pregnant rats throughout gestation with IGF-I lessened maternal constraint on fetal development (Gluckman et al. 1992) suggests that a fall in maternal plasma IGF-I caused by nutritional restriction may have an important influence on fetal growth.
(iii) IGF-II.

Maternal starvation elevated maternal but decreased fetal plasma IGF-II concentrations. Others have reported decreased fetal serum IGF-II concentrations after maternal nutritional restriction (Straus et al. 1991). Owens et al. (1989) have reported that experimentally-induced maternal hyperinsulinaemia caused a fall in both fetal plasma IGF-I and IGF-II concentrations, presumably by reducing fetal substrate supply, but maternal plasma IGF concentrations were not reported in these studies. The possible influence of maternal plasma IGF-I on fetal growth has already been mentioned but the different plasma response of the IGFs may suggest that IGF-II has a different role in the maternal circulation. In the lamb IGF-II has been reported to block the effect of IGF-I in stimulating protein synthesis (Koea et al. 1992) so perhaps the rise in maternal plasma IGF-II concentration represents a similar blocking action on maternal IGF-I activities.

The percentage fall in fetal plasma IGF-II concentrations on starvation was not as great as that in fetal plasma IGF-I concentrations. It may be that the proportionately lesser effect on fetal plasma IGF-II concentration is due to the large amount of IGF-II in the fetal circulation that is associated with the circulating type 2 receptor (Gelato et al. 1989) which was only moderately affected by maternal starvation. The IGF binding capacity of fetal plasma IGFBP-2 also increased on starvation and because this IGFBP has a much higher affinity for IGF-II than IGF-I (Gallaher et al. 1992), starvation may have less effect on fetal plasma IGF-II concentrations. IGFBP-3 binds both IGFs with equal affinity but decreases on starvation (Gallaher et al. 1992) and it has been reported that a decrease in circulating IGFBP-3 concentration leads to accelerated clearance of plasma IGF-I (Davenport, D'Ercole & Underwood 1990). Because IGF-II is normally present in much higher concentrations than IGF-I in fetal sheep plasma and because it is a stronger ligand for most IGFBPs, a reduction in plasma IGFBP-3 and type-2 receptor concentrations on starvation should increase the plasma ratio of free IGF-II to IGF-I and so cause IGF-II to out-compete IGF-I for the remaining plasma IGF-binding capacity. Following carunclectomy (Owens et al. 1991), mild growth retardation of fetal sheep is associated with reduced fetal plasma IGF-I but not IGF-II concentrations. However,
in the same studies where fetal growth retardation is more severe, fetal plasma IGF-II concentrations are reduced. These findings may suggest that more severe or prolonged undernutrition may be required for fetal plasma IGF-II concentrations to fall further than we observed. The fact that fetal plasma IGF-II concentrations were more resistant to maternal starvation than IGF-I concentrations and the observation that fetal plasma IGF-II concentrations were only depressed in severe fetal growth retardation suggests that IGF-II may have a more constitutive role in fetal growth than IGF-I. Studies in mice provide more direct evidence that IGF-II is of greater importance in at least embryonic development (Nicoll et al. 1991).

Glucose infusion to fetuses restored fetal plasma IGF-II concentrations to control values suggesting glucose has an important role in the plasma regulation of both of the IGFs. The observation that maternal plasma IGF-II responses to starvation were different to fetal responses and that maternal plasma IGF-II concentrations remained unchanged during glucose infusion to the fetuses suggests that fetal plasma IGF-II concentrations, like those of IGF-I, are also regulated by fetal factors independently of maternal factors. The importance of fetal plasma insulin in fetal plasma IGF-II regulation is suggested by the data but will be examined more thoroughly in the next chapter.

(iv) IGFBPs.

Fetal plasma IGFBP responses to starvation were the qualitatively similar to those in the mother. During starvation maternal and fetal plasma IGFBP-1 and BP-2 levels increased, whereas maternal and fetal plasma IGFBP-3 levels decreased. Maternal plasma IGFBP-4 levels also decreased during starvation while fetal plasma IGFBP-4 levels were unchanged. The fall in blood glucose, plasma insulin and IGF-I concentrations on starvation are likely causes of the changes seen in the plasma levels of at least some of the IGFBPs and these factors feature prominently in postnatal studies on the regulation of circulating IGFBP concentrations (reviewed by Clemmons and Underwood, 1991). Some clues to fetal plasma IGFBP regulation are found in the results of the glucose infusion studies and the insulin infusion experiments in the next chapter.
IGFBP-1.

The increase in maternal and fetal plasma IGFBP-1 levels on starvation was consistent with the concomitant increase in fetal blood glucose and plasma insulin concentrations. Glucose and insulin have both been reported to counter-regulate circulating concentrations of IGFBP-1 as measured by RIA (Suikkari, Koivisto, Rutanen, Järvinen, Karonen & Seppälä 1988; Lewitt & Baxter 1988). Endocrine IGFBP-1 has been suggested to have an important role in blood glucose regulation (Lewitt & Baxter 1991). The increase in maternal and fetal plasma IGFBP-1 levels on starvation may counter insulin-like effects of the IGFs.

Glucose infusion to fetuses rapidly returned fetal plasma IGFBP-1 levels to control values. Osborn et al. (1992) report that physiological replacement of glucose to 72-h starved ewes caused similar effects on maternal and fetal plasma levels of IGFBP-1 and hepatic IGFBP-1 mRNA expression. The influence of insulin on plasma IGFBP-1 regulation will be discussed further in Chapter 5. There are likely to be insulin-independent influences of glucose on plasma IGFBP-1 regulation because fructose, which is able to stimulate glycolysis but not insulin-mediated hexose uptake, also counter-regulates plasma IGFBP-1 concentrations when infused into humans (Snyder & Clemmons 1990). Further evidence for an insulin-independent role for glucose in the regulation of plasma IGFBP-1 concentration is provided by the observation that adenylate cyclase inhibitors have an additive effect to hexose-uptake inhibitors in blocking IGFBP-1 counter-regulation by glucose (Lewitt & Baxter 1990).

IGFBP-2

Fetal plasma IGFBP-2 levels increased during starvation and since IGFBP-2 is the predominant fetal IGFBP (Zapf et al. 1990; McCusker et al. 1991a; Gallaher et al. 1992) this increase may have some physiological significance. The most probable effect of the increased plasma IGFBP-2 levels may be to block anabolic actions of the IGFs as these may be inappropriate during maternal starvation when fetal substrate supply is low. Inhibitory effects of IGFBP-2 on IGF activity have been reported (Ross et al. 1989).
The mechanisms underlying the increase in fetal plasma IGFBP-2 levels are less clear as IGF-I treatment is reported to increase plasma IGFBP-2 levels in adult humans (Zapf et al. 1990). As fetal plasma IGFBP-2 levels were only slightly reduced by glucose infusion to the fetuses, and the resultant increase in plasma insulin concentration, glucose and insulin would appear to have little influence on the regulation of circulating IGFBP-2. A lack of influence of insulin on the level of plasma IGFBP-2 is consistent with findings in human studies (Clemmons, Snyder & Busby 1991). Fetal plasma IGFBP-2 levels were close to control levels after 48 h maternal refeeding which may suggest metabolic factors other than glucose and insulin are involved in fetal plasma IGFBP-2 regulation. Protein restriction has been reported to increase hepatic IGFBP-2 mRNA expression in rats although restriction in caloric intake had minimal effects (Straus & Takemoto 1990). Thus, decreases in amino acid uptake on starvation may be responsible for our observations.

**IGFBP-3.**

The decrease in fetal plasma IGFBP-3 level on starvation parallels the fall in plasma IGF concentrations and may suggest one of the most important roles of endocrine IGFBP-3 is as an IGF carrier. This possibility may appear to be in conflict with earlier suggestions about the role of IGFBP-2. However, as discussed earlier the reduced levels of fetal plasma IGFBP-3 may result in a proportionately greater fall in plasma IGF-I, rather than IGF-II concentration. This in turn this may have important implications if endocrine IGF-I has a role in protein anabolism as suggested (Harding et al. 1992).

The fall in plasma IGFBP-3 levels on starvation has been demonstrated in postnatal studies and GH and IGF-I have been cited as major regulators (Clemmons, Thissen, Maes, Ketelslegers & Underwood 1989). Experiments on transgenic mice with altered expression of GH and IGF-I suggest that IGF-I can induce IGFBP-3 independently of GH (Camacho-Hubner et al. 1991) so the changes in fetal plasma IGF-I concentrations may be responsible for changes in plasma levels of IGFBP-3 during starvation. However, circulating levels of IGFBP-3 as determined by ligand blot are markedly depressed in Laron type dwarfs (Cotterill et al. 1992). Further, IGF-I treatment of
Laron dwarfs had no influence on plasma IGFBP-3 levels (Rosenfeld et al. 1992) suggesting that GH is the most important regulatory factor. Recent studies suggest that GH has some role in fetal growth (Mesiano et al. 1989; Gluckman et al. 1992a) so GH may also feature in fetal IGFBP-3 regulation. The number of the putative oPL receptors has been reported to fall in fetal sheep liver on 72 h maternal starvation but then increases on maternal refeeding (Freemark et al. 1989). This may actually represent a fall and rise in fetal liver oGH receptor number if they are the same receptor as has been suggested by Klempt et al. (1993). Thus, a fall in fetal oGH receptor number on starvation may lead to GH resistance and a subsequent fall in fetal plasma IGFBP-3 levels. The slow return of fetal plasma IGFBP-3 to control levels on maternal refeeding may represent an increase in fetal oGH receptor number and GH sensitivity.

**IGFBP-4.**

Fetal plasma IGFBP-4 levels did not change during starvation but increased on glucose infusion to the fetuses while maternal plasma IGFBP-4 levels were markedly influenced by starvation. There are conflicting reports regarding the postnatal role of IGF-I in the regulation of IGFBP-4 in vitro (Ceda et al. 1991; Neely et al. 1992). In our studies the changes seen in maternal and perhaps fetal plasma IGFBP-4 levels would be compatible with IGF-I having a positive influence on circulating IGFBP-4 levels. The possible influence of insulin on levels of fetal plasma IGFBP-4 will be discussed in Chapter 5.

**(v) Circulating Type 2 Receptor.**

The fall in the plasma concentration of the type 2 receptor during starvation may suggest that its major role is as an IGF-II carrier. There are no reports describing other roles for the circulating form of the type 2 receptor.

A fall in fetal plasma type 2 IGF-II receptor concentrations following maternal starvation and the small increase on glucose infusion to the fetuses may also reflect changes in the membrane bound form of the receptor. There are studies that suggest insulin has a role in determining the ratio of membrane associated and free receptor, with more of the free form of the receptor present in high insulin conditions.
(Clairmont & Czech 1990; 1991). These observations are supported by
our data but as the circulating type 2 receptor had not returned to
control values on glucose infusion or maternal refeeding there must also
be factors other than insulin involved in its regulation.

In the maternal circulation IGF-II receptor concentrations, when
detected, were 10-fold lower than fetal concentrations, which is similar
to the relationship found in other species (Kiess et al. 1987; Gelato et al.
1989).

(vi) PL

Replacement of glucose to the fetus at physiological
concentrations during maternal starvation did not have the same marked
effect on fetal plasma PL concentrations as did glucose infusion to ewes
at pharmacological concentrations (Chapter 3, Oliver et al. 1992). In
other studies where physiological rates of glucose infusion have been
given to 72-hr starved pregnant ewes there were no reported increases in
fetal plasma PL concentration, and indeed fetal plasma PL concentration
fell from starved values (Freemark et al. 1989). These findings suggest
that physiological replacement of glucose reverses starvation-induced
increases in fetal plasma PL concentrations by increasing numbers of
putative PL receptors which will in turn remove receptor resistance
(Freemark et al. 1992).

Infusion of the amino acid mixture to the fetuses had no effect on
fetal plasma PL concentrations despite the fact that postnatally infusion
of arginine (Handwerger et al. 1978) and ornithine (Handwerger et al.
1981) caused increases in maternal plasma PL concentrations. There was
no apparent explanation for the effect of the amino acids on plasma PL
concentrations in the ewe and the authors ruled out the possible influence
of insulin release because of negative results from separate insulin
infusion experiments (unpublished). The lack of influence of the mixed
amino acid infusate on fetal plasma PL concentrations does not preclude
the possibility that different concentrations of certain amino acids may
have different effects.

In summary, infusion of glucose to fetuses of starved ewes
returned fetal plasma IGF-I concentrations to near starved values but
infusion of amino acids was without effect. The glucose infusion to fetuses also returned fetal plasma IGF-II concentrations to near control values and these studies suggest that the plasma concentrations of both fetal IGFs are regulated by fetal glucose independently of maternal metabolic and hormonal factors. The degree to which fetal insulin was involved in fetal plasma IGF regulation by glucose was not clear from these studies. Fetal glucose replacement had mixed effects on the plasma levels of fetal plasma IGFBPs and the plasma concentrations of the type 2 receptor but was particularly effective in decreasing the levels of plasma IGFBP-1 to control values. The effect of glucose infusion on fetal plasma PL concentrations appeared to depend on the amount infused but there is also the possibility that maternal and fetal glucose infusion may have different effects.
CHAPTER 5. EFFECT OF FETAL INFUSION OF INSULIN ON BLOOD METABOLITE, PLASMA IGF, IGFBP, INSULIN AND PL CONCENTRATIONS.

INTRODUCTION.

The data from Chapter 4 suggest that fetal glucose is an effective regulator of fetal plasma IGF-I and IGF-II concentrations. Infusion of glucose at physiological rates to fetuses of starved ewes returned fetal plasma IGF-I and IGF-II concentrations to near control values. Fetal plasma insulin concentrations also returned to control values during the fetal infusion of glucose, so it was not clear whether the effect of glucose on the IGFs was direct, or mediated by insulin. To answer this question we used the same protocol described in the last chapter but instead of infusing glucose to the fetuses, we infused insulin.

Insulin has been suggested as an important regulator of circulating IGFBP concentrations postnatally (Clemmons and Underwood 1991) and it is possible that some of the suggested effects fetal glucose infusion had on fasted levels of fetal plasma IGFBPs (Chapter 4) were due to glucose-stimulated insulin release. Fetal infusion of insulin should indicate if insulin is involved in the influence of fetal glucose on the fetal plasma levels of IGFBPs.

MATERIALS AND METHODS.

(i) Maternal Starvation/Fetal Insulin Replacement Protocol.
Recombinant human insulin (Eli Lilly, Indianapolis, USA, 0.17 U bolus and a total dose of 2 U over 24 h) was infused into fetuses after 48 h maternal starvation (n = 4). At the conclusion of the infusion mothers were fed ad libitum for 48 h. The similar protocol design to those of Chapter 4 allows some comparison of results and also allows Chapter 3 observations from the last day of starvation to be used for comparison. The insulin was mixed with 5 ml maternal plasma and made up to a volume of 30 ml with physiological saline. Maternal plasma was included in the infusate to minimise adsorption of insulin to the syringe and infusion line. For the same reason, the infusion line was flushed with the stock solution of insulin (100 U/ml) prior to loading the
infusate. These methods used to reduce the loss of insulin by adsorption resulted in insulin concentrations of infusate from the infusion syringe and infusion line being the same as that required for the infusion dose. Fetal blood glucose concentration was measured at least every 30 min during the first 4 h of the insulin infusion to ensure that values did not fall to a point where fetal death could occur. Also, because the insulin infusion was likely to stimulate oxidative metabolism of fetal substrates, fetal pH and pO₂ were monitored at the same time as fetal blood glucose. At no point in any of the studies did the insulin infusion rate have to be slowed because of extreme fetal hypoglycaemia, acidosis or hypoxia. The same sampling protocol used for the glucose and amino acid infusion (Chapter 4) was used for these experiments. Paired maternal and fetal blood samples were taken daily in the morning before normal feeding time and in the afternoon 8 h later. On the day of the fetal infusion additional samples were taken 4 h before and immediately before the start of the infusion (0 h), and then at +2, +4, +8 and +24 h of the infusion. The ewes were refed ad libitum for 48 h after the completion of the infusions.
RESULTS.
(i) Metabolites.
Starvation.
Fetal and maternal blood glucose and lactate responses were similar to those observed for the glucose and amino acid infusion

Maternal and Fetal Blood Glucose Responses to Maternal Starvation, Fetal Infusion of Insulin and Maternal Refeeding.

![Graph showing maternal and fetal blood glucose responses to maternal starvation, fetal insulin infusion, and maternal refeeding.](image)

Figure 5a. Values are means ± SD, calculated for the change from mean baseline blood glucose concentration for each animal. Starvation began at 0 h. The solid bar indicates i.v. insulin infusion to the fetus (n = 4).
groups in Chapter 4 (Fig. 5a, 5b and Table 5a). Fetal blood AN concentrations did not change significantly during starvation (Fig. 5c and Table 5a).

Maternal and Fetal Blood Lactate Responses to Maternal Starvation, Fetal Infusion of Insulin and Maternal Refeeding.

Figure 5b. Values are means ± SD, calculated for the change from mean baseline blood lactate concentration for each animal. Starvation began at 0 h. The solid bar indicates i.v. insulin infusion to the fetus (n = 4).
Insulin Infusion.

None of the measured fetal and maternal blood metabolite concentrations changed significantly during fetal infusion of insulin (Fig. 5a, 5b, 5c and Table 5a).

Maternal and Fetal Blood AN Responses to Maternal Starvation, Fetal Infusion of Insulin and Maternal Refeeding.

Figure 5c. Values are means ± SD, calculated for the change from mean baseline blood AN concentration for each animal. Starvation began at 0 h. The solid bar indicates i.v. insulin infusion to the fetus (n = 4).
Maternal Refeeding.

Fetal and maternal blood glucose concentrations returned to control values during 48 h maternal refeeding (Fig. 5a and Table 5a).

Table 5a. Blood Metabolite Responses to Maternal Starvation, Insulin Infusion to the Fetus and Maternal Refeeding.

<table>
<thead>
<tr>
<th></th>
<th>Glucose (mM)</th>
<th>Lactate (mM)</th>
<th>AN (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FETAL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.0 ± 0.1</td>
<td>0.6 ± 0.3</td>
<td>7.7 ± 0.8</td>
</tr>
<tr>
<td>48 h Starved</td>
<td>0.6 ± 0.2*</td>
<td>0.5 ± 0.3</td>
<td>8.5 ± 0.5</td>
</tr>
<tr>
<td>24 h Infusion</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.5</td>
<td>7.7 ± 0.9</td>
</tr>
<tr>
<td>48 h Refeeding</td>
<td>1.0 ± 0.2*</td>
<td>0.7 ± 0.3</td>
<td>7.6 ± 0.8</td>
</tr>
<tr>
<td><strong>MATERNAL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.4 ± 0.3</td>
<td>0.3 ± 0.1</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>48 h Starved</td>
<td>1.4 ± 0.5*</td>
<td>0.2 ± 0.1</td>
<td>4.2 ± 0.9</td>
</tr>
<tr>
<td>24 h Infusion</td>
<td>1.4 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>48 h Refeeding</td>
<td>2.5 ± 0.4*</td>
<td>0.4 ± 0.3</td>
<td>4.4 ± 0.4</td>
</tr>
</tbody>
</table>

Blood concentrations (mM) are means ± SD (n = 4). Control, last measurement before starvation; 48 h Starved, value after 48 h starvation; 24 h Infusion, value after 24 h infusion to the fetus; 48 h Refeeding, value after 48 h maternal refeeding. *, Significantly different from previous value (p < 0.05). AN; amino nitrogen.

(ii) IGF-I.

Fetal and maternal plasma IGF-I concentrations fell during maternal starvation (Fig. 5d and Table 5b). Fetal plasma IGF-I concentrations increased during fetal infusion of insulin and were the same as control concentrations by the end of maternal refeeding (Fig. 5d and Table 5b). Maternal plasma IGF-I concentrations remained depressed during insulin infusion but slowly increased during maternal refeeding.
Maternal and Fetal Plasma IGF-I Responses to Maternal Starvation, Fetal Infusion of Insulin and Maternal Refeeding.

Figure 5d. Values are means ± SD, calculated for the change from mean baseline plasma IGF-I concentration for each animal. Starvation began at 0 h. The solid bar indicates i.v. insulin infusion to the fetus (n = 4).

(iii) IGF-II.
Fetal plasma IGF-II concentrations fell during maternal starvation, while maternal plasma IGF-II concentrations did not change significantly (Fig. 5e and Table 5b). Fetal plasma IGF-II concentrations did not change during fetal infusion of insulin, but returned to control values during maternal refeeding. Maternal plasma IGF-II
concentrations did not change during insulin infusion or maternal refeeding.

**Maternal and Fetal Plasma IGF-II Responses to Maternal Starvation, Fetal Infusion of Insulin and Maternal Refeeding.**

![Graph showing change in plasma IGF-II concentrations](image)

Figure 5e. Values are means ± SD, calculated for the change from mean baseline plasma IGF-II concentration for each animal (n = 4). Filled bars, maternal responses. Open Bars, fetal responses. Starved, value after 48 h starvation; Infused, value after 24 h infusion to fetus; Refed, value after 48 h maternal refeeding. *, significantly different from previous value (p < 0.05).

**Table 5b. Plasma Hormone Responses to Maternal Starvation, Insulin Infusion to the Fetus and Maternal Refeeding.**

<table>
<thead>
<tr>
<th></th>
<th>IGF-I (nM)</th>
<th>IGF-II (nM)</th>
<th>Insulin (nM)</th>
<th>PL (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FETAL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>13.0 ± 1.7</td>
<td>143.7 ± 37.2</td>
<td>0.5 ± 0.2</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>48 h Starved</td>
<td>6.3 ± 1.5*</td>
<td>112.9 ± 15.5*</td>
<td>0.3 ± 0.1*</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>24 h Infusion</td>
<td>9.0 ± 1.1*</td>
<td>105.2 ± 16.7</td>
<td>0.6 ± 0.4</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>48 h Refeeding</td>
<td>11.7 ± 3.0</td>
<td>156.2 ± 39.8*</td>
<td>0.4 ± 0.1</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td><strong>MATERNAL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>34.2 ± 10.4</td>
<td>79.0 ± 23.5</td>
<td>1.0 ± 0.3</td>
<td>7.2 ± 1.3</td>
</tr>
<tr>
<td>48 h Starved</td>
<td>19.8 ± 9.9*</td>
<td>87.0 ± 34.9</td>
<td>0.1 ± 0.0*</td>
<td>14.5 ± 5.6</td>
</tr>
<tr>
<td>24 h Infusion</td>
<td>13.2 ± 8.0*</td>
<td>70.0 ± 10.1</td>
<td>0.1 ± 0.0</td>
<td>13.9 ± 5.0</td>
</tr>
<tr>
<td>48 h Refeeding</td>
<td>22.7 ± 8.6*</td>
<td>58.1 ± 7.0</td>
<td>1.2 ± 0.9</td>
<td>13.6 ± 3.5</td>
</tr>
</tbody>
</table>

Plasma concentrations (nM) are means ± SD (n = 4). Control, last measurement before starvation; 48 h Starved, value after 48 h starvation; 24 h Infusion, value after 24 h infusion to the fetus; 48 h Refeeding, value after 48 h maternal refeeding. *, Significantly different from previous value (p < 0.05).
(iv) Insulin.

Fetal and maternal plasma insulin concentrations fell during maternal starvation (Fig. 5f and Table 5b). Fetal plasma insulin concentrations returned to control values during 24 h of fetal infusion.

**Maternal and Fetal Plasma Insulin Responses to Maternal Starvation, Insulin Infusion to the Fetus and Maternal Refeeding.**

Figure 5f. Values are means ± SD, calculated for the change from mean baseline plasma insulin concentration for each animal. Starvation began at 0 h. The solid bar indicates i.v. insulin infusion to the fetus (n = 4).
of insulin and remained there during maternal refeeding. Maternal plasma insulin concentrations remained depressed during insulin infusion but returned to control values during maternal refeeding.

**Maternal and Fetal Plasma IGFBP-1 and BP-2 Responses to Maternal Starvation, Fetal Infusion of Insulin and Maternal Refeeding.**

*Figure 5g.* Plasma IGFBP levels expressed as mean % change from control (± SD, n = 4). *Filled bars,* maternal responses. *Open Bars,* fetal responses. Starved, value after 48 h starvation; Infused, value after 24 h infusion to fetus; Refed, value after 48 h maternal refeeding. *,* significantly different from previous value (p < 0.05).
(v) IGFBPs.

Fetal and maternal plasma IGFBP-1 and BP-3 responses to starvation as determined by ligand blot were similar to those described for the glucose infusion groups of Chapter 4. (Fig. 5g, 5h and Table 5c). However, during starvation maternal and fetal plasma IGFBP-2 levels did not increase significantly and fetal plasma IGFBP-4 levels

**Maternal and Fetal Plasma IGFBP-3 and BP-4 Responses to Maternal Starvation, Fetal Infusion of Insulin and Maternal Refeeding.**

![Graph showing changes in IGFBP-3 and IGFBP-4 levels](image)

**Figure 5h.** Plasma IGFBP levels expressed as mean % change from control (± SD, n = 4). Filled bars, maternal responses. Open Bars, fetal responses. Starved, value after 48 h starvation; Infused, value after 24 h infusion to fetus; Refed, value after 48 h maternal refeeding. * significantly different from previous value (p < 0.05).
fell. Fetal plasma IGFBP-1 levels fell to control from starved values when insulin was infused to the fetus (Fig. 5g and Table 5c). Maternal plasma IGFBP-1, BP-2 and BP-3 levels were unaffected by fetal infusion of insulin. Fetal plasma IGFBP-2, BP-3 and BP-4 levels were unaffected by fetal infusion of insulin (Fig. 5g, 5h and Table 5c). Maternal plasma IGFBP-4 levels continued to fall during insulin infusion. During 48 h maternal ad libitum refeeding plasma levels of maternal and fetal IGFBP-1, BP-2, BP-3 and BP-4 were near control values.

**Table 5c. Maternal and Fetal Plasma IGFBP Responses to Maternal Starvation, Insulin Infusion to the Fetus and Maternal Refeeding.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>48 h Starved</th>
<th>24 h Infused</th>
<th>48 h Refed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FETAL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP-1</td>
<td>1.0</td>
<td>1.41 ± 0.10*</td>
<td>0.96 ± 0.10*</td>
<td>0.90 ± 0.10</td>
</tr>
<tr>
<td>BP-2</td>
<td>1.0</td>
<td>1.10 ± 0.08</td>
<td>1.03 ± 0.10</td>
<td>1.08 ± 0.18</td>
</tr>
<tr>
<td>BP-3</td>
<td>1.0</td>
<td>0.71 ± 0.16*</td>
<td>0.65 ± 0.02</td>
<td>0.93 ± 0.16*</td>
</tr>
<tr>
<td>BP-4</td>
<td>1.0</td>
<td>0.86 ± 0.06*</td>
<td>1.04 ± 0.16</td>
<td>0.96 ± 0.14</td>
</tr>
<tr>
<td><strong>MATERNAL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP-1</td>
<td>1.0</td>
<td>1.18 ± 0.12*</td>
<td>1.15 ± 0.14</td>
<td>0.90 ± 0.10*</td>
</tr>
<tr>
<td>BP-2</td>
<td>1.0</td>
<td>1.21 ± 0.26</td>
<td>1.16 ± 0.20</td>
<td>0.98 ± 0.08*</td>
</tr>
<tr>
<td>BP-3</td>
<td>1.0</td>
<td>0.85 ± 0.06*</td>
<td>0.72 ± 0.10</td>
<td>0.85 ± 0.08*</td>
</tr>
<tr>
<td>BP-4</td>
<td>1.0</td>
<td>0.75 ± 0.06*</td>
<td>0.62 ± 0.06*</td>
<td>0.80 ± 0.12*</td>
</tr>
</tbody>
</table>

Plasma IGFBP levels are expressed relative to control values (means ± SD, n = 4). Control, last measurement before starvation; 48 h Starved, value after 48 h starvation; 24 h Infused, value after 24 h infusion to the fetus; 48 h Refed, value after 48 h maternal refeeding. *, Significantly different from previous value (p < 0.05).

(vi) Circulating IGF-II Receptor.

Plasma type 2 receptor concentrations were not measured in these studies.

(vii) PL.

Insulin infusion to the fetus had no significant effect on fetal or maternal plasma PL concentrations (Table 5b).
DISCUSSION.

(i) Metabolites.

The reason why fetal infusion of insulin had little effect on fetal blood glucose concentrations may be that as insulin-induced fetal glucose uptake increased more glucose was delivered by facilitated diffusion to the fetus from the mother. In this way the insulin infusion may have indirectly increased placental glucose transfer to the fetus as also suggested by other studies (Jodarski et al. 1985).

Blood lactate responses in the insulin-infused group of fetuses were similar to those seen in the glucose-infused fetuses providing further support for the suggestion that fetal glucose uptake determines fetal lactate production (Sparks et al. 1982).

Fetal blood AN concentrations did not change significantly during the course of these experiments. Possible reasons why there were no significant changes in fetal blood AN concentrations on maternal starvation, fetal infusion of insulin or maternal refeeding may have been the low number of animals studied and high inter-animal variation in blood AN response. Infusion of insulin to fetal sheep has previously been demonstrated to decrease total fetal blood amino acid concentrations (Philipps et al. 1990). Fetal infusion of insulin produced more marked effects on the whole blood concentration of some individual amino acids as compared to others (Philipps et al. 1990) so it is possible in our studies, where total blood AN concentrations were measured, the effects of fetal insulin on some amino acids may have been masked.

(ii) IGF-I.

Infusion of insulin to the fetus increased fetal plasma IGF-I concentrations from starved values suggesting insulin may indeed be important in the regulation of fetal IGF-I (Oliver, Harding, Breier & Gluckman 1994, in press). This is consistent with previous findings (Gluckman et al. 1987) where pancreatectomy suppressed both plasma insulin and IGF-I concentrations while the fetus was hyperglycaemic. However in that study it was not possible to conclude that plasma IGF-I regulation by insulin was glucose-independent because the reduction in insulin-stimulated glucose uptake (Fowden, Silver & Comline 1986)
could also have contributed. In situations where glucose supply is normal, fetal hyperinsulinaemia leads to increased fetal plasma somatomedin-like activity (Hill & Milner 1980; Heinze et al. 1982; Spencer et al. 1983) and plasma IGF concentrations (Susa & Schwartz 1985). In streptozotocin-diabetic adult rats insulin replacement returned hepatic IGF-I mRNA transcription to near control values (Pao et al. 1992), but this effect could also have been mediated by increased glucose uptake. It is apparent from pancreatectomy and subsequent insulin replacement experiments (Fowden et al. 1989) that insulin is required for the maintenance of fetal growth and perhaps this is achieved in part by insulin-induced maintenance of circulating IGF-I concentrations as well as promotion of substrate uptake by insulin.

(iii) IGF-II.

Insulin replacement did not restore fetal plasma IGF-II concentrations (Oliver et al. 1994, in press). This suggests there is a clear difference in the regulation of fetal plasma IGF-I and IGF-II concentrations. Possible explanations for these observations are not easy to find. In the pancreatectomised sheep fetus plasma IGF-II concentrations are increased along with those of plasma glucose despite the fact that fetal plasma insulin and IGF-I concentrations are suppressed (Gluckman et al. 1987). There is a possibility IGF-II data from this earlier study may be flawed because of the rat receptor assay technique used. However the observed fetal plasma IGF-II responses are compatible with results from our studies. Together they suggest that the regulation of plasma IGF-II concentrations are not linked to glucose-stimulated insulin release, as may be the case with fetal plasma IGF-I concentrations, but perhaps to extracellular glucose concentrations or different mechanisms of cellular glucose uptake.

IGF-II is reported to have a major role in embryonic and early fetal growth in mice (DeChiara et al. 1990; Nicoll et al. 1991). If this is true in the sheep in early pregnancy at a time when the fetal insulin response to glucose may not be developed (Shelly, Bassett & Milner 1973; Houghton, McDonald & Challis 1989) then insulin independence of IGF-II regulation may be important to maintain growth. In early gestation substrate availability is also not as likely to be restricted as in late gestation and thus rapid changes in circulating IGF-II
concentrations in response to nutrition-induced insulin changes may be less critical. In contrast, the increase in fetal plasma IGF-I concentrations throughout gestation (Gluckman & Butler 1983) may reflect the increasing influence of GH and insulin in the development of fetal plasma IGF-I regulation.

(iv) IGFBPs.

During starvation maternal and fetal plasma IGFBP levels from the insulin-infused group responded in a similar fashion to those of the glucose infused-group. The main difference was that during starvation fetal plasma IGFBP-4 levels as measured by ligand blot fell in the insulin infusion group. Because of the lack of knowledge regarding the regulation of circulating concentrations of IGFBP-4 it is difficult to explain the difference between the two groups of fetuses but the higher variability of plasma IGFBP-4 responses to starvation in the glucose infusion group of fetuses may have been an important factor. The effects of fetal infusion of insulin on plasma IGFBP levels were similar to those obtained from the glucose infusion experiments (Gallaher, Oliver, Eichhorn, Kessler, Kiess, Harding, Gluckman & Breier 1994, in press). The most pronounced effect of insulin infusion was again on plasma levels of IGFBP-1, with increases on starvation being reversed. It is likely that the effects of glucose on fetal plasma IGFBP-1 levels (Chapter 4) were mediated by insulin although some insulin-independent effects of glucose on IGFBP-1 regulation have been described (Snyder & Clemmons 1990; Lewitt & Baxter 1990). Hepatic IGFBP-1 mRNA has been reported to be transcriptionally regulated by insulin in streptozotocin-diabetic rats (Pao et al. 1992) but this influence of insulin does not exclude the possibility that insulin-mediated intracellular glucose uptake was responsible. Apart from effects on hepatic IGFBP-1 mRNA expression there are other possible mechanisms to explain the fall in fetal plasma IGFBP-1 levels on insulin infusion. Bar et al. (1990) reported that insulin stimulated movement of IGFBP-1 from vasculature to tissues in the isolated rat heart, so perhaps a similar effect is occurring in the insulin-infused sheep fetus. From both the glucose and insulin infusion studies it would appear that fetal plasma levels of IGFBP-1 are the most responsive of the IGFBPs to changes in metabolic state and this is compatible with the suggested role of circulating IGFBP-1 in blood glucose regulation (Lewitt & Baxter 1991).
(v) PL.

In Chapter 3 it was reported that maternal of infusion of glucose raised fetal blood glucose 2 to 3 fold above normal concentrations, and in response fetal plasma oPL concentrations increased markedly. The reason for the increase in plasma oPL concentration was not clear from the study but increased concentrations of blood glucose, lactate or plasma insulin during the infusion may have been responsible. High insulin concentrations in vitro stimulate PL release from human term placental explants and this effect may have been mediated by the IGF-I receptor (Bhaumick et al. 1987). During the initial stages of the insulin infusion studies fetal plasma insulin concentrations were comparable to those obtained following the maternal glucose infusion (Chapter 3) but no change in fetal plasma PL concentrations were observed. IGF-I infusion to fetuses, which also elevated fetal IGF-I plasma concentrations 2-3 fold over a similar period of time, caused no change in fetal plasma PL concentrations (Harding, unpublished observations). In other unrelated umbilical cord occlusion experiments in our laboratory fetal blood lactate concentrations were obtained that were similar to those obtained after the maternal infusion of glucose, but again there was no effect on fetal plasma PL concentrations (Johnson, unpublished observations). An early in vitro study on human term placenta explants demonstrated a direct PL-releasing action of glucose at physiological concentrations but as reported in Chapter 4 infusion of glucose at physiological rates to the sheep fetus had no effect on fetal plasma PL concentration. There is no clear explanation for the rapid increase in fetal plasma PL concentration seen after infusion of glucose, at pharmacological rates, to fasted ewes.

In summary, insulin infusion to the fetus demonstrated that fetal insulin is an important mediating factor in the regulation of fetal plasma IGF-I concentration by fetal glucose. However, the increase in fetal plasma IGF-II concentrations from starved values during glucose infusion to fetuses (Chapter 4) appears to be regulated by insulin-independent mechanisms. Fetal plasma IGFBP-1 levels fell rapidly from fasted values on fetal infusion of insulin. This suggests that similar effects on plasma IGFBP-1 levels after glucose infusion may at least in part be due to mediation by glucose-induced insulin release in the fetus.
The marked response of fetal plasma PL concentrations to maternal infusion of glucose reported in Chapter 3 do not appear to be mediated by fetal insulin.
CHAPTER 6. THE EFFECT OF FETAL INFUSION OF oPL ON BLOOD METABOLITE, PLASMA IGF-I, IGF-II, INSULIN AND PL CONCENTRATIONS.

INTRODUCTION.

Infusion of glucose to starved ewes at pharmacological rates caused a sharp increase in fetal plasma PL concentrations (Oliver et al. 1992; Chapter 3). However the effects of PL infusion to the fetus on fetal blood glucose concentration has not been investigated. In in-vitro experiments PL has been shown to influence fetal glycogen metabolism (Freemark & Handwerger 1984, 1985 & 1986) and amino acid uptake (Hill et al. 1986). PL has also been reported to have a role in the regulation of the IGFs (Hurley et al. 1977; Adams et al. 1983; Pilistine, Moses & Munro 1984; Hill et al. 1985; Swenne et al. 1987; Singh et al. 1992). To study the possible influences of PL on the circulating concentrations of fetal glucose, lactate, AN, IGF-I and -II we have infused late gestation sheep fetuses with recombinant oPL.

MATERIALS AND METHODS.

(i) Fetal Infusion of PL Protocol.

Recombinant oPL (Genentech, San Francisco, USA, 100 μg bolus then 500 μg over 24 h) was infused into 5 fetuses. A design suiting paired within-animal comparison was used in order to conserve scarce oPL and to minimise animal usage. The experiment consisted of three 24 h infusions; saline, oPL then saline again. Paired maternal and fetal blood samples (2 ml) were taken 2 h before the start of each infusion and at 0, +1, +2, +4, +8, +12, +22 and +24 h of infusion. Because fluctuations in circulating metabolites are likely to influence plasma IGF concentrations, two days prior to and during the study the daily feed intake was divided into 6 portions given at 4 h intervals. Ideally the period of nutritional conditioning may have been longer (Mellor 1987) but with the strategy used diurnal variations in plasma IGF concentration were not high.
RESULTS.

(i) Metabolites and Fetal Condition.

Infusion of oPL to fetuses had no significant effects on maternal or fetal blood glucose and lactate concentrations (Fig. 6a, 6b and Table 6a). Fetal blood AN concentrations fell on fetal infusion of oPL.

Maternal and Fetal Blood Glucose Responses to Fetal Infusion of PL.

![Blood glucose responses](image)

**Figure 6a.** Blood concentrations (mM) are means ± SD.
6. Fetal Infusion of oPL.

Maternal and Fetal Blood Lactate Responses to Fetal Infusion of PL.

Figure 6b. Blood concentrations (mM) are means ± SD.

while maternal blood AN concentrations did not change significantly (Fig. 6c and Table 6a). Fetal blood AN concentrations slowly returned to control values during the second saline infusion. Fetal pH, blood gases and haematocrit did not change significantly during the course of the experiment.
Maternal and Fetal Blood AN Responses to Fetal Infusion of oPL.

Figure 6c. Blood concentrations (mM) are means ± SD. *, grouped values significantly different than those of previous infusion period ($p < 0.05$).

(ii) IGF-I, IGF-II and Insulin.

Infusion of oPL to fetuses did not affect maternal or fetal plasma IGF-I, IGF-II or insulin concentrations (Fig. 6d, 6e, 6f and Table 6b).
Table 6a. Blood Metabolite Concentrations During Saline and PL Infusions to Fetuses.

<table>
<thead>
<tr>
<th></th>
<th>Glucose (mM)</th>
<th>Lactate (mM)</th>
<th>AN (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FETAL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline Infusion #1</td>
<td>1.0 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>7.3 ± 1.1</td>
</tr>
<tr>
<td>PL Infusion</td>
<td>0.9 ± 0.3</td>
<td>0.9 ± 0.4</td>
<td>6.6 ± 0.4*</td>
</tr>
<tr>
<td>Saline Infusion #2</td>
<td>0.9 ± 0.4</td>
<td>0.9 ± 0.4</td>
<td>6.8 ± 0.5</td>
</tr>
<tr>
<td><strong>MATERNAL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline Infusion #1</td>
<td>2.7 ± 0.7</td>
<td>0.4 ± 0.2</td>
<td>4.7 ± 1.2</td>
</tr>
<tr>
<td>PL Infusion</td>
<td>2.7 ± 0.8</td>
<td>0.3 ± 0.1</td>
<td>4.2 ± 1.0</td>
</tr>
<tr>
<td>Saline Infusion #2</td>
<td>2.8 ± 0.9</td>
<td>0.3 ± 0.1</td>
<td>4.2 ± 1.0</td>
</tr>
</tbody>
</table>

Values are means ± SD for 7 metabolite measurements obtained throughout each infusion period. *, significantly different from previous infusion period (n = 5, p < 0.05).

Table 6b. Plasma Hormone Concentrations During Saline and PL Infusions to Fetuses.

<table>
<thead>
<tr>
<th></th>
<th>IGF-I (nM)</th>
<th>IGF-II (nM)</th>
<th>Insulin (nM)</th>
<th>PL (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FETAL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline Infusion #1</td>
<td>11.0 ± 3.7</td>
<td>128.5 ± 31.6</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>PL Infusion</td>
<td>10.1 ± 3.8</td>
<td>130.5 ± 29.3</td>
<td>0.3 ± 0.1</td>
<td>3.3 ± 1.1*</td>
</tr>
<tr>
<td>Saline Infusion #2</td>
<td>9.2 ± 3.4</td>
<td>130.5 ± 43.8</td>
<td>0.3 ± 0.1</td>
<td>1.1 ± 0.9*</td>
</tr>
<tr>
<td><strong>MATERNAL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline Infusion #1</td>
<td>25.9 ± 5.6</td>
<td>74.8 ± 21.3</td>
<td>0.8 ± 0.7</td>
<td>6.8 ± 2.4</td>
</tr>
<tr>
<td>PL Infusion</td>
<td>25.2 ± 7.9</td>
<td>75.3 ± 27.5</td>
<td>1.0 ± 1.0</td>
<td>6.9 ± 2.4</td>
</tr>
<tr>
<td>Saline Infusion #2</td>
<td>24.6 ± 8.5</td>
<td>95.1 ± 47.5</td>
<td>0.8 ± 0.6</td>
<td>7.4 ± 3.2</td>
</tr>
</tbody>
</table>

Values are means ± SD for 7 hormone measurements obtained throughout each infusion period. *, significantly different from previous infusion period (n = 5, p < 0.05).

(iii) PL.

Fetal plasma oPL concentrations increased 8-fold during the oPL infusion and had returned to baseline values 12 h into the second saline infusion with an apparent half-life of 3 h (Fig. 6g and Table 6b). Maternal plasma oPL concentrations were unaffected by infusion of oPL to fetuses.
Maternal and Fetal Plasma IGF-I Responses Fetal Infusion of PL.

Figure 6d. Plasma concentrations (nM) are means ± SD.
Maternal and Fetal Plasma IGF-II Responses to Fetal Infusion of PL.

Figure 6c. Plasma concentrations (nM) are means ± SD.
Maternal and Fetal Plasma Insulin Responses to Fetal Infusion of PL.

![Graph showing plasma insulin concentrations in maternal and fetal samples](image)

**Figure 6f.** Plasma concentrations (nM) are means ± SD.
Maternal and Fetal Plasma PL Responses to Fetal Infusion of PL.

Figure 6g. Plasma concentrations (nM) are means ± SD. *, grouped values significantly different from those of previous infusion period \( (p < 0.05) \).
DISCUSSION.

(i) Metabolites.

Fetal blood AN concentrations fell in response to infusion of oPL to fetuses. This effect may reflect an increase in fetal amino acid consumption, but a different experimental design using tracer studies would have to be used to address this possibility. There was no noticeable change in maternal blood AN concentration although any change may have been masked by the considerable inter-animal variation in values. The lack of change in maternal blood AN concentrations may suggest that it is unlikely that oPL infusion stimulated any large change in placental transfer of amino acids. In human fetal fibroblasts and myoblasts hPL stimulates amino acid uptake, although the presence of an antibody to IGF-I slightly impaired the response suggesting PL-stimulated IGF-I release may have been partly responsible (Hill et al. 1986). In our studies there was no change in fetal plasma IGF-I, IGF-II or insulin concentrations on oPL infusion indicating that the action of oPL on fetal blood AN concentrations may have been direct. However, there is the possibility that a paracrine effect of oPL on IGF-I release may have been at least partly responsible for the fall in fetal blood AN concentrations. There is also the possibility that PL may indirectly influence IGF bioactivity by altering the circulating concentration and modulating influence of the IGFBPs. The influence of infusion of PL to fetuses on the circulating concentrations of the IGFBPs has yet to be determined.

Receptor binding studies suggest that there are 2 different classes of PL/GH receptor in the fetal sheep liver (Freemark et al. 1987). One class of hepatic receptor binds oGH and oPL with equal affinity while a second class of receptor that is predominant in fetal life binds oPL with much higher affinity than oGH. Similar receptors with high affinity for oPL have been demonstrated in ovine fetal fibroblasts (Fowlkes & Freemark 1992). In fetal rat diaphragm oPL stimulated amino acid transport, while oGH and rGH were without effect suggesting the effect was unique to PL (Freemark & Handwerger 1983). These studies may suggest that the influence of infused PL on fetal blood AN concentrations in our study was mediated by receptors with higher affinity for oPL. The effect of GH infusion on metabolism in fetal sheep awaits testing.
Fetal blood glucose concentration was not affected by an 8-fold elevation in fetal plasma PL concentrations. PL promotes glycogen synthesis in fetal rat (Freemark & Handwerger 1984) and sheep hepatocytes in vitro (Freemark & Handwerger 1986) so a large elevation in fetal plasma PL concentrations in vivo may have been expected to cause fetal blood glucose concentration to fall as increased amounts of glucose are sequestered into glycogen. However fetal glucose consumption was not measured. Since the maternal circulation represents a large pool of glucose that is available to the fetus by facilitated diffusion across the placenta, any increased use of fetal glucose for glycogen synthesis could be accommodated without necessarily causing a noticeable fall in circulating fetal blood glucose concentrations. The influence of PL on glycogen synthesis in vivo has yet to be tested using a suitable experimental design.

In contrast to the suggested anabolic actions of PL in the fetus, in the mother PL has been reported as having substrate-mobilising effects (Thordarsen et al. 1987) and also a role in promoting insulin resistance (Ryan & Enns 1988). We did not see any evidence for these effects in the fetus, in that there was no increase in fetal blood glucose, AN and plasma insulin concentrations during oPL infusion. The putative PL receptor with higher affinity for oPL than oGH is reported to be more predominant in the fetus than in the mother (Freemark et al. 1987) and is suggested to mediate some anabolic actions of oPL in the ovine fetal liver (Freemark & Handwerger 1986). Receptors that bind oGH and oPL with equal affinity are reported to be more common in the pregnant ewe (Freemark et al. 1987) so perhaps this class of receptor mediates the substrate-mobilising activities of PL in the mother. These observations may explain why PL appears to have different roles in the mother and fetus.

(ii) IGFs

Infusion of oPL to fetuses had no detectable effect on fetal plasma IGF-I and IGF-II concentrations despite the fact there have been numerous fetal in vitro (Adams et al. 1983; Hill et al. 1985; Strain et al. 1987; Swenne et al., 1987) and postnatal in vivo studies (Hurley et al. 1977; Pilistine et al. 1984; Singh et al. 1993) that suggest PL has an
important role in IGF regulation. There are several possible reasons why we observed no effect of oPL infusion on fetal plasma IGF concentrations. In many of the above studies, PL from a different species was used to test for IGF-releasing activity. PL structure is not highly conserved between species (Colosi et al. 1989; Warren et al. 1990), so it is possible that in the studies where oPL was injected into hypophysectomised (Hurley et al. 1977) or GH-deficient rats (Singh et al. 1992) that the observed effects on plasma IGF-I concentrations were due to oPL mimicking the effect of rGH. The same may be true in the other rat studies where hPL was used. The use of recombinant oPL in our experiment rather than purified oPL excludes the possibility of contaminant GH variants causing changes in plasma IGF concentrations. Another possible reason for the lack of influence of infused oPL on fetal plasma IGF concentrations may be that receptors with high affinity for oPL do not mediate any such activity. Plasma IGF-I concentrations markedly increase during infusion of bGH to pregnant ewes whereas in the same study oPL was without effect (Breier, unpublished observations). This may suggest that the IGF-I-releasing effects of bGH were mediated via binding to receptors with higher affinity for oGH rather than the higher affinity oPL receptors. The effects of GH infusion on fetal plasma IGF-I concentrations have yet to be tested. Finally, other possible reasons for the lack of effect of PL infusion on fetal plasma IGF concentrations may have been the dose of oPL used or perhaps at the gestation at which the experiments were performed, fetal responsiveness to oPL was low.

(iii) PL

The plasma half-life of the infused oPL was approximately 3 h. The slow plasma disappearance of the infused oPL may suggest there is a circulating PL binding protein present similar to the macroglobulin PL-binding complex reported in rodents and man (Southard & Talamantes 1989), but as yet there is no direct evidence for such a binding protein in sheep.

Because the placenta is the only source of PL, placental size may be a determinant of the circulating concentrations of PL. In ewes where PL was measured during a control period maternal plasma PL concentration and total placentome weight were moderately correlated
(r² = 0.31, p = 0.007) but there was no correlation between fetal plasma PL concentration and total placentome weight. In Chapter 3 it was noted that maternal plasma PL concentration tended to be higher in ewes on a low plane of nutrition. Placentome morphology and the influence of long term nutritional status at the time of implantation may also be important determinants of plasma PL concentrations.

The infusion of oPL into fetal sheep did not produce many of the results expected; there were no effects on fetal blood glucose, lactate and plasma IGF concentrations. It may be that these effects would be seen at higher doses, but the plasma PL concentrations obtained by these infusions were at the top of the range seen physiologically and the hormone was tested in the homologous species. The same batch of oPL was very somatogenic in rats (Singh et al. 1992). The effect of the oPL infusion on fetal blood AN concentration was consistent with actions seen in earlier in vitro studies (Freemark & Handwerger 1983; Hill et al. 1986) and may point to a physiological role for PL in fetal amino acid metabolism. Receptors with high affinity for oPL may have mediated the effect on fetal blood AN concentrations but it seems unlikely that the same receptors or oPL are involved in the regulation of fetal plasma IGF-I or IGF-II concentrations.
CHAPTER 7. SUMMARY.

OVERVIEW.
Fetal growth in late gestation is largely regulated by fetal substrate supply (Owens 1991). Nutrition also is a major determinant of the circulating concentrations of the IGFs, IGFBPs (Clemmons & Undwerwood 1991) and PL (Tyson et al. 1971; Brinsmead et al. 1981; Fielder et al. 1987; Freemark et al. 1989). In turn, the IGFs and PL have been suggested to be important influences on fetal growth and metabolism (Gluckman et al. 1990; Handwerger 1991; Owens 1991), while the IGFBPs may modulate the influence of IGF-I and IGF-II (Clemmons 1990; Clemmons 1991. The main aim of these studies was to characterise the interaction between nutrition and the concentrations of IGF-I, IGF-II, IGFBPs 1 to 4 and PL in the fetal circulation.

THE INFLUENCE OF FETAL GLUCOSE SUPPLY AND INSULIN ON FETAL PLASMA IGF-I AND IGF-II.
We have previously shown that 72 h maternal starvation causes fetal plasma IGF-I concentrations to fall and that maternal refeeding causes a return of fetal plasma IGF-I concentrations to pre-starved values (Bassett et al. 1990c). A limitation of those studies was that the specific metabolic and hormonal factors that regulated fetal plasma IGF-I concentrations were not identified and we were unable to determine if fetal plasma IGF-I concentrations were regulated by fetal or maternal factors. Amino acids have important influences in the postnatal regulation of plasma IGF-I concentrations (Isley et al. 1983; Clemmons et al. 1985) but their effects on fetal plasma IGF-I concentrations had not been investigated.

We found that fetal plasma IGF-I, IGF-II and insulin concentrations increased to pre-starved values on infusion of glucose to the sheep fetus during continued maternal starvation (Oliver et al. 1993; 1994). Maternal plasma IGF-I and blood glucose concentrations remained depressed during glucose infusion to the fetus suggesting fetal plasma IGF-I concentrations were independently regulated by fetal glucose. However, infusion of an amino acid mixture to fetuses of starved ewes had no effect on fetal plasma IGF-I and insulin concentrations (Oliver et al. 1993). One explanation for the lack of
effect on fetal plasma IGF-I or insulin concentrations may be that concentrations of specific amino acids were not high enough in the infusate we used. The decrease in fetal glucose supply bought about by maternal starvation may also have had an influence as amino acid infusion to fetuses of similar gestational age has been reported to augment the fetal plasma insulin response to glucose infusion (Fowden 1980). The effect of fetal glucose supply on fetal plasma insulin and IGF-I responses to amino acid infusion could be tested by infusing an amino acid mixture to two groups of fetuses whose mothers were either well-fed or starved.

Teleologically, if IGF-I is important in the regulation of fetal growth there are several reasons why fetal glucose should be more influential in its regulation. Fetal glucose is the preferential substrate for fetal and placental metabolism during periods of adequate maternal nutrition (Battaglia & Meschia 1978). In our experiments we have observed that fetal blood glucose concentrations changed more quickly and to a larger extent than fetal blood AN concentrations in response to decreases in substrate supply, so it may be more appropriate for fetal plasma IGF-I concentrations to be regulated by glucose. There is the possibility that individual amino acids, which are more influenced by starvation (Liechty et al. 1991) may also be involved in the regulation of fetal plasma IGF-I concentrations. Because infusion of IGF-I to fetal sheep is suggested to increase fetal amino acid uptake and inhibit catabolism of amino acids the dominant influence of glucose in fetal plasma IGF-I regulation may be indirectly influence fetal amino acid metabolism (Harding et al. 1992).

Pancreatectomy and subsequent insulin replacement experiments suggest insulin has an important role in fetal growth in late gestation (Fowden et al. 1989) but the effect of insulin replacement on fetal plasma IGF-I concentrations was not investigated. Postnatally, insulin has been reported to restore hepatic IGF-I mRNA in streptozotocin-diabetic rats (Pao et al. 1992). The role of insulin in fetal IGF-II regulation at tissue or plasma level is not well characterised. Fetal plasma IGF-II concentration as measured by rat receptor assay was reported to have increased following fetal pancreatectomy (Gluckman et
(al. 1987) suggesting insulin may have different roles in the regulation of plasma IGF-I and IGF-II concentrations.

The current studies show that fetal plasma IGF-I and IGF-II concentrations fell on maternal starvation. Infusion of insulin to fetuses returned fetal plasma IGF-I, but not IGF-II concentrations to near pre-starved values (Oliver et al. 1994). This suggests plasma IGF-I and IGF-II concentrations are regulated differently in that insulin at least partially regulates fetal plasma IGF-I concentrations while fetal plasma IGF-II concentrations are regulated by glucose in an insulin-independent manner.

It is apparent that IGF-II is vital in embryonic and early fetal development of the mouse and rat (DeChiara 1990; Nicoll et al. 1991) while IGF-I assumes a greater role in fetal growth in mid to late gestation (Nicoll et al. 1991). During early gestation fetal plasma insulin concentrations may not necessarily reflect fetal substrate supply so it may be inappropriate for plasma IGF-II regulation to be mediated by insulin. However, in late gestation the fetal insulin response to blood glucose concentration may be more developed (Shelly et al. 1973; Houghton et al. 1989) and therefore it may be appropriate for insulin to be an important mediator of fetal plasma IGF-I regulation. The different regulation of plasma IGF-I and IGF-II concentrations by fetal glucose and insulin may reflect their relative roles in fetal growth at different stages of gestation.

**NUTRITIONAL REGULATION OF IGFBPs 1-4.**

The nutritional regulation of endocrine IGFBPs and some of their influences on IGF bioactivity and plasma dynamics have been characterised postnatally (Clemmons 1990; Clemmons & Underwood 1991) but knowledge of their plasma regulation in fetal life is very scarce.

Our studies on plasma IGFBP responses to starvation, fetal infusion of glucose or insulin and to maternal refeeding suggest that the IGFBPs show similar nutritional regulation in the mother and fetus (Gallaher et al 1994, in press). However, during fetal infusion of glucose a small increase in fetal plasma IGFBP-4 level was observed but
there was no maternal plasma IGFBP-4 response to elevated glucose during maternal refeeding.

The response of fetal plasma IGFBP-1 levels during starvation and subsequent fetal infusion of glucose or insulin suggests that the theory that endocrine IGFBP-1 has a role in glucose counter-regulation (Lewitt & Baxter 1991) may be extended to the late gestation fetus. Modulation of the insulin-like activities of the IGFs may be especially important in the late gestation fetus when glucose supply is more likely to be restricted. This may also be an important role for IGFBP-2 as the predominant IGFBP in the fetal circulation. The increased fetal plasma IGFBP-2 level and perhaps IGF binding capacity during maternal starvation may help explain the observation that fetal plasma IGF-I concentrations increased during IGF-I infusion to fetuses of underfed ewes, but did not increase in fetuses of well fed ewes. During maternal starvation and refeeding plasma IGFBP-3 levels exhibited similar trends in mother and fetus. The main function of IGFBP-3 in fetal life may be as an IGF carrier. Plasma IGFBP-4 levels appear to be regulated differently by nutrition in mother and fetus and our results do not indicate what role this IGFBP may have in fetal life.

**NUTRITIONAL REGULATION OF THE CIRCULATING TYPE 2 IGF RECEPTOR.**

The nutritional regulation of the circulating type 2 IGF receptor has not been reported previously. In common with other studies we found that fetal plasma concentrations of the receptor were much higher than maternal values (Gelato et al. 1989; Gallaher et al. 1994). Fetal plasma concentrations of the receptor fell during maternal starvation (Gallaher et al. 1994).

Because plasma concentrations of IGF-II are high in fetal life (Gluckman & Butler 1983) and free IGF-II has a short plasma half life (Bassett et al. 1990b) this may suggest that in the fetal circulation the type 2 IGF receptor, along with IGFBP-2, has an important function as a plasma IGF-II carrier protein. The fetal plasma concentrations of the receptor were higher when the fetus had high blood glucose and plasma insulin concentrations. This finding is consistent with the postnatal observation that insulin promotes release of the type 2 receptor into the
circulation (Clairmont & Czech 1990; 1991) but the direct effect of insulin on the type 2 receptor has yet to be examined in fetal life.

**THE NUTRITIONAL REGULATION AND ENDOCRINE ACTIONS OF oPL.**

Plasma PL concentrations have been reported to increase significantly in the fetal sheep following 72 h maternal starvation (Freemark et al. 1989). Subsequent infusion of glucose to the ewe at physiological rates returned fetal plasma PL to pre-starved concentrations (Freemark et al. 1989). The authors suggest that fetal plasma PL is inversely regulated by fetal glucose supply and PL in turn counter-regulates the number of putative PL receptors in the fetal liver. The influence of the nutritional status of the ewe prior to starvation had not been investigated.

In our studies fetal plasma PL concentrations increased on 72 h maternal starvation in fetuses whose mothers had originally been on a high level of feed intake whereas there was no increase in fetal plasma PL concentrations in fetuses whose mothers had originally been on a low level of feed intake (Oliver et al. 1992). The period of underfeeding may have caused PL receptor numbers to be down regulated to such an extent that the relationship between fetal plasma PL and blood glucose concentrations and fetal hepatic PL receptor number was disrupted and so fetal plasma PL concentrations became unresponsive to the effects of maternal starvation. Infusion of glucose to ewes at a rate that increased fetal blood glucose concentration 3-fold caused a marked increase in fetal plasma PL concentration (Oliver et al. 1992) and this response was in marked contrast to the effect of maternal infusion of glucose at a lower rate (Freemark et al. 1989). Thus fetal plasma PL concentrations may respond to both low and excessively high glucose supply. The increase in fetal plasma PL concentrations on starvation suggests PL may have substrate-releasing actions as has been suggested postnatally (Thordarsen et al. 1987). However, the increase in fetal plasma PL concentrations in response to greatly increased glucose supply may reflect stimulation of anabolic activity such as glycogen synthesis in fetal liver (Freemark & Handwerger 1984 & 1986). The influences of varied fetal glucose supply on the induction of catabolic or
the anabolic actions of PL have yet to be examined in the fetus *in vivo* using labelled glucose and amino acids.

The partial redundancy of the GH-IGF axis in fetal life (Gluckman & Butler 1985; Mesiano *et al.* 1989) has led to speculation that PL acts as a 'fetal growth hormone' and has an important role in fetal plasma IGF regulation (Handwerger *et al.* 1991). However, until now recombinant PL has not been tested *in vivo* in the fetus of the homologous species.

Infusion of recombinant oPL into sheep fetuses increased fetal plasma oPL concentrations to the top of the range seen physiologically but we found no effect on fetal plasma IGF-I or IGF-II concentrations. The results suggest that oPL at the dose tested does not influence plasma IGF regulation in the fetal sheep. This is consistent with postnatal studies where infusion of oPL into pregnant ewes also had no effect on maternal plasma IGF concentrations, whereas infusion of bGH caused an increase in maternal plasma IGF-I concentrations (Breier, unpublished observations). As yet the effect of oGH infusion, or larger doses of oPL, on plasma IGF regulation have not been examined in the fetal sheep. Earlier reports that oPL increased plasma IGF-I concentrations in the postnatal rat *in vivo* (Hurley *et al.* 1977; Singh *et al.* 1992) may not be representative of the role of PL in plasma IGF-I regulation because oPL and rodent PL have low structural homology (Colosi *et al.* 1989). The observation that hPL stimulates IGF-I release from human fetal cells *in vitro* (Hill *et al.* 1985; Swenne *et al.* 1987) may represent paracrine effects of PL on IGF regulation that may not influence circulating concentrations of PL. Possible paracrine influences of oPL on IGF-I release in fetal sheep tissue remain to be investigated.

Fetal infusion of oPL caused a fall in fetal blood AN concentrations. *In vitro* incubation of human fetal cells with hPL and an IGF-I antibody substantially inhibited the effect of hPL on amino acid uptake suggesting that hPL-stimulated IGF-I release accounted for most of the activity (Hill *et al.* 1986). Even though fetal plasma IGF-I concentration did not change on infusion of PL it is possible that paracrine release of IGF-I could occur and mediate the effect of oPL on fetal blood AN concentrations. To investigate whether oPL has a direct
endocrine effect on protein metabolism in the fetal sheep, fetal consumption of labelled amino acids could be measured during oPL infusion after the influence of IGF-I had been reduced by immunoneutralisation. However, the investigation of possible paracrine effects of oPL on IGF release from ovine fetal tissue \textit{in vitro} may be the best initial approach in answering this question.

\textbf{CONCLUSION.}

These studies have further characterised the plasma regulation and functional significance of the IGF peptides, IGFBPs and PL. Glucose rather than amino acid infusion to the fetus increased fetal plasma IGF-I concentrations during continued maternal starvation. This is consistent with the hypothesis that fetal glucose supply determines amino acid usage in fetal and placental metabolism. The co-regulation of fetal plasma insulin and IGF-I concentrations in the glucose infused fetuses suggested that insulin may have a role in plasma IGF-I regulation and this hypothesis gained further support when insulin infusion to the substrate-restricted fetal sheep increased fetal plasma IGF-I concentrations. However, we found that fetal plasma IGF-II regulation was glucose dependent, but not insulin dependent. The IGFs are suggested as having important roles in fetal growth and their regulation by fetal substrate supply supports this role. The different regulation of plasma IGF-I and IGF-II concentrations by glucose and insulin may reflect different importance of each IGF at different stages of gestation.

We found that plasma IGFBP levels were similarly regulated by nutrition in the adult and fetus and confirmed that IGFBP-2 is the predominant IGFBP in the fetal circulation. Our data suggest that the IGFBPs may have similar roles in fetal and postnatal life as plasma IGF carriers and modulators of IGF bioactivity. Nutrition influenced circulating levels of the IGFBPs and therefore may influence their function. Similarly, the circulating type 2 receptor may be an important influence on the circulating concentration and bioactivity of IGF-II in fetal life.

We have demonstrated that the fetal plasma oPL response to starvation depends on the prior nutritional status of the ewe and also
that the fetal response to glucose replacement is dependent on the amount of glucose infused. Fetal plasma IGF-I, IGF-II and insulin concentrations were not affected by oPL infusion to the fetus but oPL may have a role in fetal protein metabolism.

Fetal substrate supply is the most important factor in the regulation of fetal growth. Endocrine factors such as IGF-I, IGF-II, IGFBPs, insulin and PL have been suggested as important mediators in the control of fetal growth and we have demonstrated that their plasma concentrations are regulated by fetal substrate supply. This thesis provides further evidence for the hypothesis that the interactions between fetal substrate supply and IGF-I, IGF-II, IGFBPs, insulin and PL may be central to the regulation of fetal growth.
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