Ovine placental lactogen and insulin-like growth factor-I: a study of their biological actions and potential to enhance animal production

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ABSTRACT

Ovine placental lactogen (oPL) has been considered to be important in the regulation of fetal lamb growth. Recent sequence studies have also shown that oPL has considerable structural similarity to ovine prolactin (oPRL) and ovine growth hormone (oGH), raising the possibility that oPL may have potential as a stimulator of milk yield and postnatal growth. Evidence from laboratory animals indicates that the biological actions of GH and perhaps PL are mediated largely by IGF-I, acting in both a paracrine/autocrine and an endocrine manner. However, the potential of IGF-I therapy has not been fully explored in domestic animals. Therefore, the overall aim of this study was to examine the biological actions of recombinant oPL and IGF-I and to evaluate their potential use to stimulate performance of farm animals.

In the first three experiments, the biological actions of recombinant oPL were examined in pregnant and lactating ewes, and in young growing lambs, and compared with those of bovine growth hormone (bGH). bGH treatment for 7 days from day 101 of pregnancy increased total uterus weights, but administration of an identical dose of oPL had no effect. Similarly, exogenous bGH over 5 days increased milk yields in lactating ewes, whereas such an effect was not apparent with oPL treatment. However, the reverse situation occurred in young growing lambs in which oPL, not bGH, treatment for 21 days from day 3 of life stimulated growth rate and voluntary feed intake. Different biological actions of bGH and oPL in pregnant and lactating ewes were associated with a situation in which bGH, but not oPL, treatment increased circulating concentrations of IGF-I. Conversely, in young growing lambs, the growth-promoting effect of oPL appeared to be mediated primarily via a change in voluntary feed intake since both oPL and bGH treatment had only small effects on plasma IGF-I concentrations.

The inability of bGH to stimulate plasma IGF-I concentrations in young lambs, accompanied by the lack of a growth-promoting effect of bGH, suggested that hepatic GH receptors (GHR) may not be fully functional at this stage. This hypothesis was examined in an experiment in which the ontogeny of hepatic GHR was assessed by measuring IGF-I responsiveness to a GH stimulus, as well as hepatic GH binding, in lambs of different ages (days 6/7, 20/21, 34/35, and 62/63 of life, and yearlings). Results

showed that the plasma IGF-I response to exogenous bGH in young lambs was much lower than that in yearling sheep. Consistent with this, measurement of hepatic GHR at day 7 and 63 of life, and in yearling sheep, showed that bGH treatment failed to alter GHR number in young lambs, while up-regulating it in yearling sheep. In contrast, non-stimulated GHR numbers were similar across ages. These data suggest that hepatic GHR in young lambs are not fully functional, which may explain the lack of a growth-promoting effect of bGH in newborn animals.

The importance of the GH-IGF-I axis in the regulation of post-natal growth was further demonstrated in an experiment, in which anabolic effects of recombinant IGF-I over a prolonged period (8 or 12 weeks) were measured in energy-restricted sheep. IGF-I treatment elevated circulating concentrations of IGF-I, but depressed plasma GH concentrations. The reduction in circulating GH levels was accompanied by a down-regulation of hepatic GHR. As a result, recombinant IGF-I had little growth-promoting effect although it improved other parameters such as nitrogen digestibility and components of the immune system.

In conclusion, the present study suggests that recombinant oPL, like GH, has potential in improving farm animal production. However, the biological actions of oPL seem to be mediated in a different manner from those of GH. This could have practical implications in situations where GH has no biological actions. For example, in very young lambs in which hepatic GHR are not fully functional, oPL could provide an alternative means to stimulate growth. This could be also true for IGF-I because, in young animals, the negative-feedback regulation of both plasma GH and hepatic GHR concentrations by IGF-I treatment may be less likely to limit a growth response than is the case in older animals.

EMENDATIONS

Page/Line	Thesis Reads	Should Read
47/7	the perfused mammary gland	mammary explants
95 & 96	(reverse panels for Figures 4.1 and 4.2)	
99/7	Figure 4.4	data not shown
108/Table 5. 1	Insulin	Bovine insulin
152/publication 5	Currie J M	Currie M J

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LIST OF ABBREVIATIONS

ADG	Average daily gain
AIB	Aminoisobutyric acid
ALS	Acid-labile subunit
AUC	Area under the curve
b	Bovine
BP	Binding protein
BrdU	5-Bromo-2'-deoxyuridine
CB154	Bromocriptine
CP	Crude protein
DM	Dry matter
FCR	Feed conversion rate
h	Human
hp	Human pancreatic
GH	Growth hormone
GHR	Growth hormone receptor
GRF	Growth hormone-releasing factor
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IGFBP	Insulin-like growth factor binding protein
i.m.	Intramuscular
K	The fractional decay constant
LTD	Laron-type dwarfism
LW	Liveweight
MCHC	Mean corpuscular haemoglobin concentration
ME	Metabolisable energy
MG	Mammary gland
MJ	Megajoules
M-6-P	Mannose-6 phosphate
NE	Net energy

NEFA	Non-esterified fatty acids
NSILA	Non-suppressible insulin-like activity
o	Ovine
p	Porcine
PL	Placental lactogen
PRL	Prolactin
PCV	Packed cell volume
r	Correlation
RIA	Radioimmunoassay
SE	Standard error of the mean
SRIF	Somatotropin release inhibiting factor (somatostatin)
ST	Somatotropin
TRH	Thyrotropin-releasing hormone

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CHAPTER 1: INTRODUCTION

1.1 PREAMBLE

The hormonal regulation of growth and lactation is a complex process which involves many different hormones, multiple interactions between endocrine and paracrine/autocrine control systems, and genetic and environmental factors (including nutrient availability). Growth hormone (GH), also known as somatotropin (ST), is believed to play an essential role in the hormonal component of this process.

It has been known for many years that administration of GH enhances growth rate and milk yields in farm animals, but historically it was not possible to take full advantage of this knowledge in animal production because naturally produced GH was available only in small amounts. This situation was, however, dramatically transformed in the 1970s with the development of recombinant DNA techniques which allowed GHs, specific for different animal species, to be produced in large amounts. Subsequent studies showed that recombinant GHs, like their natural analogues, improve farm animal performance (Boyd & Bauman, 1988; Enright, 1989).

More recently, recombinant DNA techniques have been used to produce biosynthetic ovine placental lactogen (oPL) and insulin-like growth factor-I (IGF-I), which may also have potential in animal production. While IGF-I is well recognized as a key hormone in the somatotrophic axis, mediating many of the actions of GH in an endocrine and paracrine/autocrine manner, it has not been studied extensively in terms of its potential to enhance productivity of animals with an intact somatotrophic axis. The potential value of placental lactogen (PL) for enhancing animal production has only recently emerged. Although oPL has more structural similarity to ovine prolactin (oPRL) than to oGH (Colosi *et al.* 1989; Warren *et al.* 1990*b*), it has been shown to exhibit somatogenic actions both *in vitro* and *in vivo.* It binds to oGH receptors in the sheep liver (Chan *et al.* 1978; Emane *et al.* 1986; Breier *et al.* 1994*a,b*) and administration of recombinant oPL stimulates weight gain in GH-deficient dwarf rats with a similar or superior potency to bovine GH (bGH) (Singh *et al.* 1992). Recombinant bPL has also been shown to have galactopoietic properties in dairy cattle (Byatt *et al.* 1992*a*). These data suggest that PL also has actions similar to those of GH.

Therefore, the objective of the experiments described in this thesis was to examine the biological actions of recombinant oPL and IGF-I and to evaluate their potential use to stimulate performance of domestic animals.

1.2 GROWTH HORMONE

1.2.1 Nature and secretion of growth hormone

Growth hormone (GH) is a peptide secreted by the anterior pituitary gland. It is structurally related to prolactin (PRL) although the two hormones exhibit a surprisingly wide diversity of biological functions. Recent sequence studies (Warren *et al.* 1990*b*) also show that placental lactogen (PL) shares considerable structural homology with GH and PRL. Such a close structural relationships among the three peptides suggest that they evolve from a common ancestral gene.

GH is secreted in a pulsatile manner in adult animals, including sheep (Cataldi *et al.* 1994). The pulsatile pattern of GH secretion is associated with the intermittent exposure of the pituitary gland to two hypothalamic peptides, GH-releasing factor (GRF) and somatostatin (or somatotropin release inhibiting factor, SRIF) which are stimulatory and inhibitory respectively. The physiological importance of the pulsatile nature of GH secretion remains unclear. However, there is evidence that growth disorders in man may be associated with altered pulsatility of GH (Blatt *et al.* 1984). Furthermore, pulsatile delivery of GH, mimicking the endogenous plasma GH pattern, stimulates greater growth rates in hypophysectomized rats compared to continuous delivery of GH (Isgaard *et al.* 1988; Bick *et al.* 1992). These data may indicate that the pulsatile nature of GH secretion is important for optimal body growth.

The pulsatile nature of GH secretion may start as early as mid-gestation in the fetal lamb, and is characterized by a markedly exaggerated pulsatile release in sheep (Gluckman, 1986) and cattle (Reynolds *et al.* 1990). Such an exaggerated pulsatile GH release is associated with elevated plasma concentrations of GH in both the ovine and bovine fetus compared to those during postnatal life (Bassett & Gluckman, 1986; Gluckman *et al.* 1986; Reynold *et al.* 1990) and has been suggested to be a consequence of the immaturity of the hypothalamo-pituitary axis (de Zegher *et al.* 1989*a,b*; Silverman *et al.* 1989). As gestation advances, with the maturation of hypothalamo-pituitary axis,

plasma GH concentrations decline, with further reductions after birth. Both amplitude and interpulse values fall so that at birth the secretory pattern is not markedly different from that of the adult (Gluckman *et al.* 1986). Similar developmental changes in the hypothalamo-pituitary axis during the perinatal period have been reported in other species including pigs (Farmer *et al.* 1992) and horses (Davicco *et al.* 1993).

The pulsatile pattern of GH secretion also shows sexual differentiation in a number of species, where pulse amplitude is higher and baseline values lower in the male than in the female (Gluckman *et al.* 1987). However, such a sexual dimorphism does not become apparent until after the onset of puberty, indicating that the sex steroid environment is primarily responsible for these sex-related differences. For example, in cattle, a sexual dimorphism is evident only at 12 months of age (Lee *et al.* 1991) when the circulating testosterone concentrations increase several fold in bulls (Henricks *et al.* 1988). A similar change in the postpubertal sex steroid levels modulates the pulsatile release of GH in the rat (Jansson *et al.* 1984; Jansson & Frohman, 1987). Recent study with rats showed that both the neonatal and adult sex steroid environments play an important role in generating the sexually dimorphic GH secretory pattern observed in the adult rat, and these effects are mediated at least in part by modulating the number, sensitivity to sex steroids, and gene expression, of the hypothalamic neurons that control GH secretion (Chowen *et al.* 1993).

Nutritional status and energy intake also markedly affect GH secretion. Acute or chronic undernutrition results in increased GH concentrations in most species, including man (Ho *et al.* 1988), sheep (Foster *et al.* 1989; Thomas *et al.* 1990), cattle (Breier *et al.* 1986; Houseknecht *et al.* 1988; Kirby *et al.* 1993) and pigs (Antinmo *et al.* 1978; Dubreuil *et al.* 1989). In both sheep (Thomas *et al.* 1990, 1991; Bauer *et al.* 1995) and cattle (Breier *et al.* 1986), such elevated GH concentrations during periods of nutritional deficit are associated with increased amplitude of GH pulses but with no changes in baseline concentrations of GH or pulse frequency. Such increases in levels of serum GH coincide with increases in GH gene transcription and GH content of the pituitary gland during periods of energy deficit, indicating an enhanced synthesis and secretion of GH (Kirby *et al.* 1993). Simultaneous measurement of portal GRF and SRIF concentrations showed that chronic undernutrition had no effect on mean concentrations of GRF, GRF pulse amplitude, or GRF pulse frequency, but reduced mean concentrations of SRIF to

50 % of those of sheep fed a normal diet (Thomas *et al.* 1991). This suggests that an enhanced GH secretion during diminished nutrition is due to the enhanced responsiveness of the pituitary gland to GRF via the reduced SRIF levels. There is also evidence that undernutrition causes a partial refractoriness of GH receptors in target tissues, including the liver (Breier *et al.* 1988*a*; Bass *et al.* 1991). For example, Breier *et al.* (1988*a*) showed that a low level of nutrition was associated with a marked reduction of hepatic GH receptor number in growing steers. Such a down-regulation of GH receptors is also likely to contribute to the increased plasma concentrations of GH by reducing clearance of GH, as reported previously in sheep, calves (Trenkle, 1976) and heifers (Lapierre *et al.* 1992).

1.2.1.1 Chemical structure of growth hormone

Growth hormone is a protein hormone comprising a single polypeptide chain of about 190 amino acids, containing two intrachain disulfide bridges, which has a molecular weight of 22,000 daltons. The amino acid sequence of growth hormone was first determined in humans and subsequently in other species (Chene *et al.* 1989). The amino acid composition of GH from a number of species shows considerable differences. For example, human and bovine growth hormones differ by 25% but ovine and bovine growth hormones are almost identical (Chene *et al.* 1989).

Growth hormone also exhibits some degree of micro-heterogeneity within a species (Chene *et al.* 1989). Variants of GH have been reported in human and other species (Lewis *et al.* 1978; Chene *et al.* 1989) and comprise both structural and post-translationally modified forms. The latter includes glycosylated, phosphorylated, disulfide dimers, non-covalent oligomers, N-acetylated, deamidated and cleaved forms of hGH. Structural variants in which one or more amino acid residues are missing or replaced are more common. One of the most common variants is the 20K hGH (Charrier & Martal, 1988) in which residues 32 to 46 are missing as a result of mRNA processing (DeNoto *et al.* 1981). It accounts for 10-15 % of the total pituitary store of hGH (Lewis *et al.* 1978) and a similar proportion (16 %) of this peptide has been reported in plasma after physiological stimuli such as exercise, or pharmacological stimuli as GRF (1-40), oestrogens or TRH (Baumann & Stolar, 1986). It has been suggested that the 20K and other minor GH variants form non-covalent oligomers such as 80K GH by dimerisation

or binding to protein, which is neither an albumin nor a globulin (Charrier & Martal, 1988).

Proportions of the different forms of GH vary with pathological and physiological state. For example, the small molecular weight form is the major constituent of plasma GH in young mice, while GH is present in the large form in adult and pregnant mice (Sinha, 1980). Although the physiological significance of the different forms of GH is not fully understood, these data suggest that they may reflect differences in the biological activities of the hormone.

It has been speculated that the various biological activities of GH are a reflection of the diversity of the GH molecule. Several studies have been designed to resolve this issue, mainly with respect to the human GH variants. Deamidated hGH variants had the same biological activity as native hGH (Skottner *et al.* 1988), while dimeric forms of hGH were much less active in *in vitro* bioassays (the Nb2 assay), receptor assays and radioimmunoassay (Brostedt *et al.* 1990). The 20K variant of hGH possesses the biological activities intrinsic to unmodified hGH (Lewis *et al.* 1978; Kostyo *et al.* 1985) but lacks, or has decreased, insulin-like activities in rat both *in vivo* and *in vitro* (Frigeri *et al.* 1979; Kostyo *et al.* 1985). This suggests that the region comprising amino acids 32-46 is most likely involved in the insulin-like activity.

Despite considerable progress towards understanding the structure-function relationships of GHs and related proteins from various species, the features that contribute to their biological actions remain unclear. The issue is complicated by the fact that the variants may have altered interaction with receptors rather than intrinsically lacking certain biological activities, as demonstrated by 20K hGH in the adipocyte of rats (Smal *et al.* 1986). Furthermore, biological activities of GHs and their fragments have generally been measured in heterologous systems, which are difficult to extrapolate relative to homologous systems. For example, 20K hGH does not bind to human liver GH receptors (McCarter *et al.* 1990), whereas it binds to both rat and rabbit liver (Closset *et al.* 1983; Hughes *et al.* 1983). Thus, the 20K form of hGH may be not be somatogenic in humans, although it is in rats (Kostyo *et al.* 1987).

1.2.1.2 Growth hormone releasing-factor (GRF)

GRF is a protein hormone which stimulates selective release of GH from the

anterior pituitary gland. Immunocytochemical studies with rats have shown that the majority of the GRF-immunoreactive cell bodies are located in the arcuate and medial perifornical region of the lateral hypothalamus (Merchenthaler *et al.* 1984). From these ventrolateral arcuate regions, GRF fibres innervate the external layer of the median eminence, from which the peptide is released into the portal blood.

Although the presence of GRF has been recognized since the late 1950s (Reichlin *et al.* 1976), the identification and sequencing of this peptide was made only in 1982. In that year, two research groups, one led by Guillemin (Guillemin *et al.* 1982) and the other led by Rivier (Rivier *et al.* 1982), successfully isolated and characterized GRF from human pancreatic tumors that caused acromegaly. One, described by Guillemin and his colleagues, was a 44-amino acid amide, hpGRF(1-44)-NH_2, and the other, characterized by Rivier and his coworkers, was the free acid hpGRF (1-40)-OH. The two molecules share the first 40 amino acids in the same sequence. Comparison of properties of tumor-derived GRF with purified hypothalamic GRF has shown identical biological activities including parallel GH-release dose-response curves and lack of effects on other anterior pituitary hormones. Antibodies against hpGRF are cross-reactive with a specific population of hypothalamic nerve cells (Merchenthaler *et al.* 1984), indicating similarity to hypothalamic GRF. The amino acid composition of human hypothalamic GRF is identical to that of tumor-derived GRF (Ling *et al.* 1984).

Isolation and characterization of hypothalamic GRF has been completed in other species, including cow (Esch *et al.* 1983), goat, sheep (Brazeau *et al.* 1984), pig (Bohlen *et al.* 1983) and rat (Spiess *et al.* 1983). Human hypothalamic GRF shows major homologies (93%, 89%, 89%, and 86%, respectively) when its primary structure is compared with that of the hypothalamic GRF from the porcine, bovine, caprine, and ovine species. This high homology between human and other species suggests that human GRF is likely to be active in a wide range of species.

Administration of hGRF stimulates specific release of GH in a dose-dependent manner in a variety of animal species, including sheep (Ohmura *et al.* 1984; Hart *et al.* 1985; Barenton *et al.* 1987) and cattle (Johke *et al.* 1984; Hodate *et al.* 1990). Barenton *et al.* (1987) examined characteristics of GH response to hGRF(1-44) or hGRF(1-29), administered as an intravenous injection in the lamb, in both acute and chronic conditions, and reported increased serum GH concentrations in both situations. Under

acute conditions, administration of GRF (0.1 ug/kg) evoked an immediate release of GH. The presence of a spontaneous pulse of GH just before or at the time of an injection of GRF did not impair pituitary responsiveness. Nor was pituitary responsiveness to GRF diminished even when administration of GRF (1.5 nmol/kg/day) was extended over a 21-day period. Conversely, there was a significant increase in the GH response to GRF during the course of chronic treatment, suggesting that chronic treatment of GRF with this dosage regimen may increase the sensitivity of the pituitary gland to GRF.

In the rat and human, there is some evidence that chronic exposure of the pituitary gland to GRF blunts the GH response to subsequent GRF treatment (Wehrenberg *et al.* 1984; Bilezikjian *et al.* 1986; Blumenfeld *et al.* 1989). The classical study of such a case was well documented by Wehrenberg *et al.* (1984). When hGRF (1-44) was infused continuously for 24 h, circulating GH concentrations increase 10 fold within 2 h of infusion, began to fall by 6 h, decreased slowly, and reached a nadir by 12 h. Rats infused for 24 h failed to respond to a bolus injection of GRF, whereas rats infused with saline responded with a normal increase in plasma GH concentrations. Measurement of pituitary GH content at the end of the GRF infusion showed greater than 80% depletion, suggesting that GH depletion is responsible for the blunted release observed. Alternatively, the blunted responsiveness of GH after chronic exposure to GRF may reflect a substantial down-regulation of GRF-binding sites (Bilezikjian *et al.* 1986).

GH response and sensitivity to GRF decrease with advancing age in sheep. In the fetal lamb, the GH response to GRF is greater in fetuses at 89 to 122 days of gestation than in fetuses at 127 to 145 days of gestation (De Zegher *et al.* 1989*b*). Similarly, the net increase in GH concentrations following a GRF infusion was negatively correlated with gestational age in the ovine fetus (Ohmura *et al.* 1984). Such a trend continues even in postnatal lambs, the threshold of responsiveness to GRF being greater in weaned lambs than in suckling lambs (Barenton *et al.* 1987). Similar results have been reported in newborn calves (Coxam *et al.* 1987) and older cattle (Johke *et al.* 1984). The reason for the decline in GH response with advancing age is not clear, but it may reflect an age-related changes in GRF secretion (Gelato & Merriam, 1986).

1.2.1.3 Somatostatin (SRIF)

Unlike GRF, somatostatin is distributed not only in the hypothalamus, but also in

the other tissues including other parts of the central nervous system (Muller, 1987), pancreas and alimentary tract (Arimura *et al.* 1975; King & Millar, 1979). In fact, study with rats has shown that these extra-cerebral sites account for 75% of the total body somatostatin (Patel *et al.* 1981).

SRIF was first isolated from sheep hypothalamic extracts (Brazeau *et al.* 1973) and found to be a tetradecapeptide with a disulphide bridge linking residues 3 and 14 (Burgus *et al.* 1973). Since then, multiple forms of somatostatin sharing common immunological characteristics have been identified in most somatostatin-containing tissues examined (Muller, 1987). These include somatostatin (somatostatin-14), amino-terminal extended somatostatin (somatostatin-28), and a larger form, which normally exists as a composite of 6K and 10K (Pierotti & Harmar, 1985). The relative proportions of these molecular forms vary considerably depending on the tissue. For example, whereas the hypothalamus and pancreas contain a greater proportion of somatostatin-14 than somatostatin-28 and high molecular weight somatostatin, the gut contains large quantities of somatostatin-28 (Patel *et al.* 1981). Such differences in relative amounts of immunoactive somatostatins in different tissues, together with the existence of multiple somatostatin receptors (Bruno *et al.* 1993), may indicate diverse, but tissue-specific biological roles.

SRIF has been shown to inhibit basal and GRF- or TRH-induced GH secretion from lamb pituitary cultures (Blanchard *et al.* 1987). Such an inhibitory effect of somatostatin on plasma GH has been confirmed *in vivo* where sheep were treated intravenously (Spencer *et al.* 1991*a*) or intracerebroventricularly (Fairhall *et al.* 1995) with SRIF. The inhibitory effect of SRIF is short lived, possibly because of the rapid disappearance of this peptide from circulation. GH levels rapidly rebound after the cessation of SRIF treatment (Sugihara *et al.* 1989; Spencer *et al.* 1991*a*). This coincides with rapid increases in GH gene transcription after the withdrawal of SRIF infusion (Sugihara *et al.* 1993). However, such a postinhibitory rebound effect by SRIF occurs only when spontaneous surges of GRF are present (Sugihara *et al.* 1989). These data, together with the recent observations that immunization against SRIF fails to abolish the pulsatile secretion of GH in sheep (Van Kessel & Laarveld, 1993; Magnan *et al.* 1995), suggest that SRIF may play only a minor role in episodic GH secretion, particularly in sheep (Van Kessel & Laarveld, 1993; Magnan *et al.* 1995).

1.2.2 Growth hormone receptors (GHR)

The first step of GH action is binding to a specific receptor on the surface of target cells. Growth hormone receptors (GHR) are those that bind GH more potently than prolactin and are assumed to mediate the somatogenic effects of GH. The presence of such GH receptors has been reported in a number of tissues, with major concentrations being evident in the liver and adipose tissue.

Although GHR are present as early as day 51 of gestation in hepatic tissue of the ovine fetus, GH binding to fetal liver microsomes is nonexistent or very slight (Gluckman *et al.* 1983; Freemark *et al.* 1986, 1988; Klempt *et al.* 1993). Recent study with sheep has shown that oGHR mRNA present in mid-gestation fetal liver differs structurally from the transcript present in late gestation fetal liver and adult liver (Pratt & Anthony, 1995), suggesting that GHR of the fetus may be non-functional. This non-functional GHR may be responsible for the lack of specific GH binding to ovine fetal liver. This also is consistent with the fact that GH has no effects on prenatal growth of lambs (Gluckman & Liggins, 1984; Parkes & Hill, 1985).

Marked developmental changes in GHR occur in the perinatal period. In sheep, the adult form of GHR appears just before term (Pratt & Anthony, 1995) or soon after birth (Gluckman *et al.* 1983) and rapidly increases a few days after birth to adult values (Gluckman *et al.* 1983). In cattle, bGH binding to liver membranes is very low between days 2 and 30 postnatally and then increases gradually up to day 180 (Badinga *et al.* 1991). In both species, the appearance of the GHR postnatally is associated with a rise in circulating concentrations of IGF-I and the appearance of GH-dependent growth. It has been suggested that the postnatal rise in hepatic GHRs is triggered by a parturition-related event rather than being a strictly age-related phenomenon (Breier *et al.* 1994*a*).

Several studies have shown that specific binding of GH to hepatic membranes involves two binding sites with a 10-fold different affinity (Gluckman & Breier, 1989). These two binding sites have been reported in sheep (Pell *et al.* 1990; Sauerwein *et al.* 1991), cattle (Breier *et al.* 1988*a*,*b*), pigs (Breier *et al.* 1989), rats (Baxter *et al.* 1980) and rabbits (Hughes, 1979). In cattle (Breier *et al.* 1988*a*,*b*) and sheep (Pell *et al.* 1990), the capacity of high affinity binding sites in the liver correlates with growth rate, with plasma IGF-I concentrations, and with the IGF-I response to exogenous GH. Similarly, in diabetic rats, the presence of the high affinity site is correlated with responsiveness

to GH (Baxter *et al.* 1980). In contrast, low affinity binding sites show no such correlation. Similar results have been reported also in sheep (Sauerwein *et al.* 1991) and pigs (Breier *et al.* 1989). These findings indicate that the capacity of high affinity binding sites has a functional significance in postnatal growth.

It is well recognized that chronic GH deficiency is associated with a reduction in the number of hepatic GHR. In both sheep and rabbits, hypophysectomy markedly reduces GHR number which is partially restored by GH treatment (Posner *et al.* 1980). There is increasing evidence that such an induction of hepatic GHR also occurs in animals with an intact somatotrophic axis. Chronic treatment with GH increases the number of hepatic GH binding sites in rats (Baxter *et al.* 1984) and pigs (Chung & Etherton, 1986). Similarly, in the intact sheep, chronic treatment with GH (0.15 to 0.55 mg/kg/day) markedly up-regulates the hepatic GHR in a dose-dependent manner (Sauerwein *et al.* 1991). Careful characterization of GHR by radioreceptor assay, after the pretreatment of membranes with $MgCl_2$ to remove endogenously bound hormone, showed that the increase in hepatic binding sites largely consisted of high-affinity GHR with little effect on low-affinity binding sites (Sauerwein *et al.* 1991). An increase in hepatic high affinity binding sites does not, however, occur in animals on a low plane of nutrition although GH treatment successfully increases circulating concentrations of IGF-I with no effects on growth rate in growing lambs (Bass *et al.* 1991). These data suggest that the up-regulation of the high-affinity GHR is central to the action of GH.

It appears that the pattern of GH administration modulates the induction of hepatic GHR, although direct evidence of this is restricted to the hypophysectomized rodent model (Maiter *et al.* 1988*a*; Bick *et al.* 1992). Pulsatile vs continuous GH treatment has been shown to have differential effects on hepatic GHR and plasma IGF-I concentrations (Bick *et al.* 1992). There is also evidence that duration of GH treatment is very important for the up-regulating of hepatic GHR, since single doses of GH acutely down-regulate the number of GH receptors (Maiter *et al.* 1988*b*).

In both growing lambs (Bass *et al.* 1991) and steers (Breier *et al.* 1988*a,b*), a low plane of nutrition leads to a marked reduction of hepatic GHR. Such a down-regulation of hepatic GHR is associated with reduced plasma IGF-I levels and with a lack of IGF-I response to GH in growing steers (Breier *et al.* 1988*b*). Conversely, a high plane of nutrition is associated with a marked increase in the number of hepatic GHR, indicating

that nutrition plays a major role in the regulation of hepatic GHR. Measurement of hepatic binding sites has shown that these binding sites in well-fed animals consist of both high- and low-affinity sites, whereas those of energy-restricted animals contain only the low affinity sites (Breier *et al.* 1988*a*). The rate of weight gain and plasma IGF-I concentrations correlate with the capacity of the high-affinity sites. This finding has led to a suggestion that the optimum level of nutrition is important in maximizing biological actions of GH. However, such a suggestion is not supported by subsequent study with lambs in which GH treatment failed to stimulate growth rate, despite its positive effects on both hepatic GHR and plasma IGF-I concentrations in animals on high and low levels of nutrition (Bass *et al.* 1991).

1.2.3 Role of IGF-I in growth hormone action

Since the classical study by Evans & Simpson (1931), GH treatment has been shown to have growth-promoting effects in a variety of species. However, such a response *in vivo* is quite often absent under *in vitro* conditions where addition of GH has small and inconsistent effects on growth-associated parameters (Kostyo & Isaksson, 1977). The different effects of GH under *in vivo* and *in vitro* conditions led Salmon and Daughaday (1957) to examine whether the effects of GH *in vivo* might be mediated indirectly. In series of experiments, they incubated cartilage with serum from hypophysectomized rats and compared the results to those with serum from normal (intact) rats. They found that serum from hypophysectomized animals was inactive, whereas serum from normal rats stimulated [^{35}S]sulfate uptake by 200%. Furthermore, treatment of hypophysectomized rats with GH restored the serum growth-promoting activity despite GH itself having no direct effect. These results led to the suggestion that GH effects are mediated by a GH-dependent factor in serum. This factor was designated "the sulfation factor" and later as "somatomedin".

In unrelated, but parallel studies around this time, Froesch *et al.* (1963) reported the presence of insulin-like biological activity in human serum which was not suppressed by addition of anti-insulin antibody to bioassays for insulin. The peptides responsible for this activity were initially called "non-suppressible insulin-like activity" (NSILA), later changed to "insulin-like growth factors" (IGFs) because these factors were shown subsequently to have both functional (Morell & Froesch, 1973) and structural

(Rinderknecht & Humbel, 1978*a,b*) similarities to insulin.

Purification of somatomedin-C and IGF-I showed that they had similar biological activities (Van Wyk *et al.* 1980), suggesting a possible structural similarity between these peptides. This suggestion was verified in a subsequent study in which the amino acid sequence of somatomedin-C was found to be identical to that of IGF-I (Klapper *et al.* 1983). Since then, the term "IGF" has been employed to refer to this family of peptides (Daughaday *et al.* 1987).

IGF-I has been implicated in many aspects of animal production such as growth, lactation, and reproduction. Studies with a variety of species have shown that IGF-I mediates many of the biological actions of GH through both endocrine and paracrine/autocrine mechanisms.

1.2.3.1 Chemical structure of IGF-I

The isolation of IGF-I was first performed for human beings (Rinderknecht & Humbel, 1978*a*). It is comprised of a single-chain polypeptide of 70 amino acids, containing three intrachain disulfide bridges, which has a molecular weight of 7646 daltons. IGF-I is composed of four domains: B-C-A-D. The A and B regions are similar to those of proinsulin with approximately 45% homology. The C domain of IGF-I is considerably shorter (12 amino acids) than the C peptide of proinsulin, with no homology. In addition, IGF-I has a C-terminal D peptide, which is absent in proinsulin. The first 16 residues of the B domain seem to be critical for IGF-I to bind to its carrier protein, since modification of this region in molecules such as IGF-I/insulin hybrids or mutants significantly reduces affinity for the IGF-binding protein (Bayne *et al.* 1988; Oh *et al.* 1993). In contrast, the A domain seems to play an important role in increasing affinity of IGF-I for its receptor, which results in enhanced mitogenic activity (Tseng *et al.* 1987), although some modifications of this region have also been shown to reduce affinities for the binding proteins in a recent study (Oh *et al.* 1993).

The primary structure of IGF-I has also been established in a variety of other species (Etherton, 1993). The amino acid sequence of IGF-I from several species shows that it is remarkably similar among species. For example, the primary structures of bovine, porcine and human IGF-I are identical, whereas rat IGF-I differs from human by three amino acid residues. The amino acid sequence of ovine IGF-I is also similar to that of

human IGF-I except that alanine is substituted for proline at residue 66 in ovine IGF-I (Francis *et al.* 1989). Variant forms of IGF-I have been identified within species. Des(1-3)IGF-I, which lacks the three amino acids at the N-terminus, has been identified in a variety of tissue extracts and biological fluids including fetal and adult brain (Carlsson-Skwirut *et al.* 1986; Sara *et al.* 1986), porcine uterus (Ogasawara *et al.* 1989) and bovine colostrum (Francis *et al.* 1986). Recently, it has been shown that this truncated IGF-I is naturally produced *in vivo* by a GH-dependent protease (Yamamoto & Murphy, 1995). This finding, together with the fact that no mRNA encoding a variant IGF-I protein sequence has been identified, indicates that this amino-terminus truncation results from post-translational modification of IGF-I. The affinity of des(1-3)IGF-I for the IGF binding proteins (IGFBPs) is greatly reduced due to the absence of Glu^3, one of the critical residues for IGF-I interaction with the IGFBPs (Ballard *et al.* 1987; Bagley *et al.* 1989). As a consequence of the reduced affinity for the IGFBPs, this naturally occurring variant is much more potent *in vivo* and *in vitro* than intact IGF-I (Ballard *et al.* 1987; Francis *et al.* 1992). It also has a much shorter half-life than IGF-I when administered intravenously (Ballard *et al.* 1991), probably because it is more readily excreted by the kidney.

1.2.3.2 Regulation of IGF-I synthesis and secretion

For many years, it was considered that liver was the only site of IGF-I synthesis. However, D'Ercole *et al.* (1984) reported that IGF-I is detectable in various rat tissues and that GH treatment increases tissue IGF-I mRNA concentrations. This and other studies (Orlowski & Chernausek, 1988) have also shown that tissue IGF-I concentrations increase prior to the elevation of serum IGF-I concentrations. These findings have led to the concept that IGF-I mediates many of biological actions of GH through both endocrine and autocrine/paracrine mechanisms.

The processes of synthesis and secretion of IGF-I are quite different from those of most of the classical polypeptide hormones in several aspects. First, IGF-I is synthesized by hepatic and a number of non-hepatic tissues, whereas most polypeptide hormones are synthesized only by specialised cells in endocrine glands. Second, synthesized IGF-I is readily secreted into the blood with no or little storage in the tissues, whereas most polypeptide hormones are normally stored in secretory granules before being released

into the bloodstream. Finally, IGF-I circulates in the blood bound to specific binding proteins (IGFBPs), thus having a prolonged half-life in the serum relative to free (unbound) IGF-I. In contrast, most polypeptide hormones circulate in the plasma without binding proteins, resulting in a short half-life. As a consequence, plasma concentrations of IGF-I do not undergo diurnal variation and are very stable in any given subject under constant nutritional and endocrine conditions, whereas the plasma levels of most other hormones vary rapidly and over a wide range.

Circulating concentrations of IGF-I are influenced by a number of factors (Van Wyk, 1985). GH status and nutrition are the major regulators of IGF-I synthesis and secretion. There is also some evidence that a negative feed-back regulation is operative in the GH-IGF-I axis. Therefore, these factors are considered in the next section.

1.2.3.2.1 Growth hormone

It is well established that GH is the primary regulator of IGF-I synthesis in a variety of species. In rats (Bates *et al.* 1993; Gosteli-Peter *et al.* 1994), hypophysectomy markedly reduces concentrations of IGF-I in both serum and tissues, and the concentrations are restored toward normal after GH treatment. However, the magnitude of IGF-I responses to GH varies considerably depending on the tissue. For example, Gosteli-Peter *et al.* (1994) have reported that GH-dependent IGF-I and IGF-I mRNA responses are most pronounced in the liver, skeletal muscle and white adipose tissues and less so in heart, testes, kidney, spleen, and thymus. These data suggest that GH regulates IGF-I synthesis in a tissue-specific manner.

Induction of IGF-I synthesis by GH is also evident in intact animals. In sheep (Bass *et al.* 1991; MacRae *et al.* 1991; Hua *et al.* 1993; Pell *et al.* 1993) and cattle (Cohick *et al.* 1989; Elsasser *et al.* 1989; Lemal *et al.* 1989; McGuire *et al.* 1995), GH treatment markedly stimulates circulating concentrations of IGF-I. There is a lag of 1-4 days, however, before concentrations of serum IGF-I reach their maximum values (Cohick *et al.* 1989; Elsasser *et al.* 1989; Lemal *et al.* 1989; MacRae *et al.* 1991; McGuire *et al.* 1995). Studies with sheep (Wynn *et al.* 1991) and cattle (Ronge & Blum, 1989; Enright *et al.* 1990) have also shown that these elevated concentrations of serum IGF-I do not return to baseline values until several days after termination of GH treatment. Such a belated response of serum IGF-I concentrations at the beginning and end of GH

treatment strongly indicates that the GH effect on IGF-I production is through the stimulation of *de novo* synthesis of IGF-I rather than through its release from a storage pool. This suggestion is further supported by the results from *in vitro* experiments in which IGF-I is continuously synthesized and released by the perfused rat liver (Schwander *et al.* 1983).

In sheep, acute and chronic treatment with GH is accompanied by a large increase of IGF-I mRNA in the liver (Hua *et al.* 1993; Pell *et al.* 1993), indicating that GH regulation of IGF-I occurs at least partly at the level of IGF-I gene expression. Pell *et al.* (1993) further characterized hepatic IGF-I mRNA by measuring steady state levels of the individual class 1 and 2 transcripts. Leader exon-specific and total IGF-I gene expression, using a single RNase protection assay, have shown that GH treatment only stimulates significant increases in expression from class 2 transcripts. These data suggest that GH regulates hepatic IGF-I production at transcriptional levels, primarily through class 2 transcripts. Such a class 2 transcript appears postnatally at the time of acquisition of GH responsiveness (Saunders *et al.* 1991).

1.2.3.2.2 Nutrition

Nutritional status plays a well-established role in the regulation of circulating concentrations of IGF-I in humans and rats (Thissen *et al.* 1994*a*). Such a role is also well recognized in ruminants. In sheep (Bass *et al.* 1991; Wynn *et al.* 1991; Pell *et al.* 1993; Bauer *et al.* 1995) and cattle of different physiological states (Breier *et al.* 1986; Breier *et al.* 1988*b*; Houseknecht *et al.* 1988; Elsasser *et al.* 1989; Ronge & Blum, 1989; McGuire *et al.* 1992*a*; Kirby *et al.* 1993; Henricks *et al.* 1994), restriction of feed for a prolonged period is associated with decreased serum concentrations of IGF-I. In both ruminant species (Hua *et al.* 1993; McGuire *et al.* 1995) and in the pig (Buonomo & Baile, 1991), an extreme nutritional regimen such as a fasting for several days leads to a further reduction in concentrations of IGF-I, which return towards normal concentrations after refeeding. These decreased concentrations of IGF-I occur despite elevated serum levels of GH, indicating an uncoupling of the GH-IGF-I axis during the period of diminished nutrition. This suggestion is further supported by the fact that responses of plasma IGF-I concentrations to a single GH treatment are diminished or even abolished under such conditions (Breier *et al.* 1988*b*; Houseknecht *et al.* 1988;

Elsasser *et al.* 1989; Kriel *et al.* 1992; McGuire *et al.* 1995). Such a dissociation between GH and IGF-I under conditions of diminished nutrition is associated with a down-regulation of high affinity hepatic GHR, the importance of which in postnatal regulation of IGF-I and growth was already discussed in the previous section (see section **1.2.2 Growth hormone receptors (GHR)**).

In growing lambs, increasing the nitrogen supply in the diet re-establishes the IGF-I response to GH challenge as well as increasing basal concentrations of IGF-I (Kriel *et al.* 1992). Similarly, in beef cattle, concentrations of plasma IGF-I increase linearly with increasing protein content within the high energy diet groups (Elsasser *et al.* 1989). While these data suggest that dietary protein content plays an important role in the regulation of plasma IGF-I levels, such a relationship is absent in the low energy diet animals (Elsasser *et al.* 1989). Therefore, in ruminants, both dietary protein and energy contents appear to be important in the regulation of plasma IGF-I concentrations. This situation is in contrast to that in rats, in which dietary protein content rather energy content is the primary nutritional determinant of circulating concentrations of IGF-I (Thissen *et al.* 1994*b*).

1.2.3.2.3 Feedback regulation

In vitro studies have shown that IGF-I administration decreases GH secretion from the pituitary gland of several species including sheep (Blanchard *et al.* 1987, 1988; Silverman *et al.* 1989). There is some evidence that centrally administered IGF-I is able to influence hypothalamic control of GH secretion (Berelowitz *et al.* 1981; Tannenbaum *et al.* 1983). While these data suggest that IGF-I exerts a negative feedback on GH secretion with actions at both the pituitary gland and hypothalamus, a recent study with rats (Harel & Tannenbaum, 1992) has shown that central administration of recombinant IGF-I or long R^3 IGF-I at several doses (0.5, 2, 3 and 10ug) fails to alter the pulsatile pattern of GH, GH peak amplitude, GH trough level, GH interpeak interval or mean plasma GH level. In contrast, a combination of IGF-I and IGF-II results in a marked suppression in those parameters, indicating that the negative effects of central administration of IGF-I on GH secretion observed in the earlier studies were possibly due to the impurity of IGF-I preparations. Such an inability of centrally administered IGF-I or its analogue to inhibit GH secretion has been reported in sheep (Spencer *et al.*

1991*b*; Fletcher *et al.* 1995). In contrast, intrapituitary administration of IGF-I at even smaller doses suppresses circulating concentrations of GH in sheep (Fletcher *et al.* 1995). These data suggest that the negative feedback effect of IGF-I on GH secretion is largely made by direct action on the pituitary gland. This suggestion is consistent with the conclusion from an earlier study with sheep, in which protein supplementation increased plasma IGF-I levels, while suppressing plasma GH levels without a change in the GH mRNA levels in the pituitary gland (Clarke *et al.* 1993).

1.2.4 Role of IGF-II in growth hormone action

While it is well established that IGF-I mediates many of the biological actions of GH in an endocrine or paracrine/autocrine manner, such a role is unclear with respect to IGF-II. A large body of evidence has shown that there is no close relationship between circulating concentrations of IGF-II and GH in a number of species including sheep and cattle (McGuire *et al.* 1992*b*). Such a dissociation between IGF-II and GH raises the question of whether this peptide can be classified as a somatomedin (as the term was originally defined).

1.2.4.1 Chemical structure of IGF-II

Like IGF-I, the isolation of IGF-II was first achieved in human beings (Rinderknecht & Humbel, 1978*b*). IGF-II contains 67 amino acids and has a 62 % identity in its amino acid sequence with IGF-I. Its primary structure is similar to that of IGF-I, although the connecting peptide chain (domain C) has four fewer residues (8 residues) than in IGF-I (12 residues) with significant sequence divergence. The maximum degree of homology between IGF-II and insulin occurs in the A and B regions, where IGF-II has 47 % homology with human pro-insulin. The C domain of IGF-II is much shorter than that of proinsulin (35 residues). Likewise, IGF-II retains a C-terminal extension (D domain), which is absent in proinsulin. The critical B chain residues that are necessary for interacting with the binding proteins are preserved. It is also suggested that the A domain is, like that of IGF-I, critical for triggering biological activity of IGF-II (Joshi *et al.* 1985).

The amino acid sequence of IGF-II has also been established in a variety of other species (Etherton, 1993). These studies have shown that IGF-II is, like IGF-I, highly

conserved among several species. For example, the amino acid sequence of bovine IGF-II differs from that of human by 3 amino acids, whereas rat IGF-II differs from that of human by four amino acid residues. The primary structure of ovine IGF-II also differs from human only at four positions in the amino acid sequence (Francis *et al.* 1989). These similarities of amino acid sequences of IGF-II are reflected in only slight differences in biological and immunological potencies (Francis *et al.* 1989).

Variant forms of IGF-II have also been identified (Daughday & Rotwein, 1989). The most common variant form of IGF-II has the substitution of Arg-Leu-Pro-Gly for Ser^{29} at the carboxy-terminal end of the B region and probably arises as a consequence of an error in mRNA splicing (Jansen *et al.* 1985). Perdue *et al.* (1994) reported that this form accounts for up to 10% of the total IGF-II present in human serum. The affinity of this variant of IGF-II for the IGF-I receptor is greatly reduced and, probably as a consequence, it has a low mitogenic activity (Hampton *et al.* 1989).

1.2.4.2 Regulation of IGF-II synthesis and secretion

IGF-II, like IGF-I, is synthesized and secreted by a wide variety of tissues. However, regulation of this peptide appears to be quite different from that of IGF-I. Although GH and nutrition have been shown to affect levels of IGF-II, such effects seem to be minor compared to those observed with IGF-I (McGuire *et al.* 1992*b*). Instead, developmental regulation appears to play a major role in synthesis of IGF-II. For example, in the rat, expression of the IGF-II gene is abundant in most tissues prenatally, but decreases dramatically at birth in all tissues except brain (Cohick & Clemmons, 1993). The decrease in IGF-II mRNA levels corresponds to the decrease in circulating concentrations of IGF-II. Similar developmental changes in IGF-II expression have been observed in other species, including ruminants (Boulle *et al.* 1993; Delhanty & Han, 1993; Carr *et al.* 1995) although, in contrast to the rat, significant levels of plasma IGF-II are still maintained postnatally. This has led to the concept that IGF-II has a significant role in fetal growth, but only a minor role in postnatal growth. This suggestion is further supported by recent studies with rats in which disruption of one of the IGF-II alleles by gene targeting and germ-line transmission of the inactivated IGF-II gene from male chimeras yielded heterozygous progeny that were smaller than their wild-type litter mates at birth (DeChiara *et al.* 1990).

1.2.4.2.1 Growth hormone

The regulation of circulating concentrations of IGF-II by GH is equivocal in humans (Davenport *et al.* 1988; Rechler & Nissley, 1990). A similar situation is also evident in ruminants, including cattle. Although some studies have shown a positive effect of GH on serum IGF-II concentrations (Breier *et al.* 1988*b*; McGuire *et al.* 1992*a*), such effects are absent in other studies (Davis *et al.* 1987; Vicini *et al.* 1991; McGuire *et al.* 1995). Conversely, Owens *et al.* (1990) reported that exogenous GH treatment decreased circulating concentrations of IGF-II in growing pigs. Similarly, van Buul-Offers *et al.* (1994) showed that GH treatment caused a small, but significant decrease of serum IGF-II concentrations in dwarf Snell mice. In both studies, GH treatment clearly had positive effects on serum IGF-I levels. These inconsistent results among studies suggest that IGF-II is, relative to IGF-I, less dependent on GH. This suggestion is consistent with a study in which hypophysectomy led to a fall in plasma concentrations of IGF-I, but not IGF-II, in fetal lambs (Mesiano *et al.* 1989).

1.2.4.2.2 Nutrition

Although nutrition is a key regulator of plasma IGF-I concentrations, such a role is less clear in regard to IGF-II. While some studies have reported that short-term deprivation of feed reduces circulating concentrations of IGF-II in cows (McGuire *et al.* 1995) and growing pigs (Buonomo *et al.* 1988), others have not (Davenport *et al.* 1988). Even in those studies where nutrition has significant effects on plasma concentrations of IGF-II, the responses are very small. Furthermore, it has been shown that chronic undernutrition has no effects on circulating concentrations of IGF-II in fetal and pregnant sheep (Bauer *et al.* 1995), and growing steers (Breier *et al.* 1988*b*). Thus, IGF-II is likely to be less sensitive to nutritional status than IGF-I. This suggestion is further supported by recent reports in which stage of lactation or alteration of dietary energy and protein intake had no effects on circulating concentrations of IGF-II in lactating dairy cows (Vicini *et al.* 1991; McGuire *et al.* 1992*a*).

1.2.4.2.3 Feedback regulation

There have been only a few studies on the role of IGF-II in the regulation of GH secretion. In the rat, IGF-II exerts little effect on GH secretion from the cultured anterior

pituitary gland (Simes *et al.* 1991). Similarly, Harris *et al.* (1991) reported that IGF-II did not have a modulatory role in GH release by human somatotrophinoma cells *in vitro.* Such a finding is consistent with results from a recent *in vivo* study in which central or intrapituitary administration of IGF-II had no effects on circulating concentrations of GH in ewes (Fletcher *et al.* 1995). Therefore, IGF-II is unlikely to exert a negative feedback regulation of GH secretion, unless its concentrations are pathologically high, as demonstrated in patients with tumour-induced hypoglycaemia (Ron *et al.* 1989).

1.2.5 Insulin-like growth factor binding proteins (IGFBPs)

Although IGF-I and IGF-II have significant structural similarities to insulin, the IGFs differ from insulin in one important respect. Unlike insulin, IGF-I and IGF-II circulate in plasma bound to a family of binding proteins (BPs). Both peptides circulate almost exclusively bound to the IGFBPs with little free IGF evident in plasma.

The IGFBPs were initially identified based on the their capacity to bind IGF-I and IGF-II during gel filtration chromatography (Baxter & Martin, 1989). Using this method, Zapf *et al.* (1975) reported that a radiolabeled human IGF (NSILA) preparation, when incubated with human serum, bound specifically to two protein fractions, one with high molecular weight (150,000 M_r) and the other with low molecular weight (50,000 M_r). Such a finding has, since then, been extended to other species, including sheep and cattle (Hossner *et al.* 1988), where radiolabeled IGF-I binds specifically to proteins of similar size.

Following these initial chromatographic studies, measurement of the IGFBPs has been refined with the development of specific radioimmunoassays (RIAs) and ligand blotting techniques. Although RIAs allow more rapid and accurate quantitative analysis of specific IGFBPs, ligand blotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has found widespread use where no suitable antibody against a particular IGFBP exists or if simultaneous analysis of all IGFBPs in a sample is required (Hossenlopp *et al.* 1986). Using this technique, three major binding proteins have been detected in serum from several species, although other distinct IGFBPs have recently been identified (Cohick & Clemmons, 1993). The major IGFBPs have been designed IGFBP-1 through -3 which are based on amino acid sequences.

The primary structures of IGFBP-1, 2 and 3 have recently been determined.

Comparisons of amino acid sequences show several structural similarities between the proteins (Cohick & Clemmons, 1993). Specifically all three proteins contain 18 cysteines which are spatially conserved in spite of differences in molecular weight. IGFBP-1, previously known as amniotic fluid binding protein, PP12 (Koistinen *et al.* 1986), was the first to be cloned. This protein has been purified from human amniotic fluid and decidua. The protein from human decidua consists of 259 amino acids with M_r of 25,293 (Julkunen *et al.* 1988) and can be divided into three regions based on cysteine abundance. The first region contains 12 of the cysteine molecules and its sequence is highly conserved across species. The second region contains no cysteine residues and its structure is divergent across species. The third region contains the final six cysteine molecules and is highly conserved across species. It also contains a distinctive Arg-Gly-Asp sequence (RGD) in the COOH terminal region which may function as a cell recognition signal. In addition, IGFBP-1 has a number of regions (so-called PEST regions) rich in Pro-Glu-Ser-Thr residues (Julkunen *et al.* 1988), suggesting that the intracellular half-life of IGFBP-1 may be very short.

IGFBP-2 was originally identified as a distinct binding protein produced by rat BRL 3A fetal liver cells (Mottola *et al.* 1986). The primary structure of this protein has subsequently been isolated and cloned in humans (Binkert *et al.* 1989), rats (Margot *et al.* 1989) and cattle (Szabo *et al.* 1988). Recently, the gene for ovine IGFBP-2 was also cloned (Delhanty & Han, 1992). This polypeptide is composed of 284 amino acids. Its leader sequence peptide is longer than IGFBP-1 but all 18 cysteines are conserved. Unlike IGFBP-1, its structure is much more highly conserved across species. For example, ovine IGFBP-2 amino acid sequence shows 86, 95 and 85% homology with the rat, bovine and human, respectively (Delhanty & Han, 1992). IGFBP-2 also contains an Arg-Gly-Asp sequence (RGD) in the COOH terminal region.

IGFBP-3 is the IGF-binding subunit of the 150 kD GH-dependent binding complex that is present in serum (Baxter & Martin, 1989). The native complex consists of three components: (1) IGFBP-3, an acid stable M_r 47,000-53,000 glycoprotein doublet on SDS-PAGE gel under nonreducing conditions (Martin & Baxter, 1986); (2) an acid-liable subunit (ALS) of M_r 84,000-86,000 glycoprotein doublet; and (3) IGF-I or -II. The native complex can form under neutral pH condition by combination of ALS with a preformed IGFBP-3/IGFs complex (Baxter & Martin, 1989). IGFBP-3 cannot bind to

ALS in the absence of IGFs. IGFBP-3 binds IGF-I and IGF-II with a similar affinity (Oh *et al.* 1993) although there is some evidence that one of its binding sites exclusively binds IGF-II in several species including sheep (Hodgkinson *et al.* 1991*b*) and pig (Evock *et al.* 1990). This protein is the most abundant form among IGFBPs in serum and thus carries most of the circulating IGF-I and IGF-II.

IGFBP-3 cDNA has been isolated from the human (Wood *et al.* 1988), pig (Shimasaki *et al.* 1990), rat (Albiston & Herington, 1990) and cattle (Spratt *et al.* 1991) cDNA libraries. The mature polypeptide deduced from the human, porcine, rat and bovine cDNA consists of 264, 266, 265, and 264 amino acids, respectively. IGFBP-3 has all 18 cysteines conserved but, unlike IGFBP-1 and 2, it has three potential N-linked glycosylation sites. Furthermore, it does not contain an RDG sequence. Comparison among species has shown 78-89% homology in their deduced amino acid sequences.

The regulation of the concentrations of IGFBPs has been the subject of intensive studies in recent years. Much of the earlier work has recently been reviewed by Cohick & Clemmons (1993) and Etherton (1993). Nutrition and GH are considered to be the major common regulators of these binding proteins.

Circulating concentrations of IGFBP-1 seem to be very sensitive to nutritional status. In the human, plasma IGFBP-1 levels rise during fasting and decline after meals, when insulin levels increase (Busby *et al.* 1988; Suikkari *et al.* 1988; Young *et al.* 1992). Similarly, in pregnant ewes and fetal lambs, maternal fasting for three days in late gestation significantly increases circulating concentrations of maternal and fetal IGFBP-1 (Osborn *et al.* 1992). These changes are reversed by refeeding and prevented by glucose infusion. Conversely, hyperglycaemia induced by glucose infusion in these animals suppresses plasma IGFBP-1. In both rats (Ooi *et al.* 1992) and sheep (Osborn *et al.* 1992), these changes in plasma IGFBP-1 levels are paralleled by corresponding changes in hepatic IGFBP-1 mRNA, suggesting that liver IGFBP-1 gene expression is the principal determinant of plasma IGFBP-1 levels. Plasma IGFBP-1 responses to nutritional changes occur very rapidly in monogastric species, including humans, although much slower responses have been reported in ruminants such as sheep (Osborn *et al.* 1992). For example, in the human, IGFBP-1 concentrations can be suppressed as much as 4- to 5-fold after ingestion of food and are suppressible with glucose and/or insulin infusion by several fold within a few minutes (Busby *et al.* 1988; Suikkari *et al.*

1988). This situation is reflected by the marked diurnal variation in plasma IGFBP-1 levels which are inversely associated with fluctuations in plasma insulin and glucose concentrations (Baxter & Cowell, 1987, 1989; Cohick & Clemmons, 1993). This has led to the view that IGFBP-1 plays a significant role in acute control of glucose homeostasis. This suggestion is consistent with a previous report that administration of an IGF/IGFBP-1 complex to rats blocks the hypoglycaemic response to exogenous IGF-I, while injection of IGFBP-1 alone increases plasma glucose levels (Lewitt *et al.* 1991). In addition, IGFBP-1 has been considered to be GH independent (Cohick & Clemmons, 1993). However, recent *in vitro* studies have shown that GH suppresses IGFBP-1 mRNA levels in a dose-dependent manner in rat hepatocytes (Kachra *et al.* 1994; Thissen *et al.* 1994*b*), suggesting that the expression of IGFBP-1 is not completely GH independent.

IGFBP-2 responds to nutritional changes in a similar manner to IGFBP-1. However, the speed of IGFBP-2 responses to metabolic change is markedly different from that of IGFBP-1. For example, in humans, serum IGFBP-2 levels significantly increase only after prolonged fasting (9 days) (Clemmons *et al.* 1991). This situation is reflected in a less dynamic fluctuation in plasma IGFBP-2 concentrations after a meal or glucose infusion compared with IGFBP-1 (Clemmons *et al.* 1991). A similar situation is also apparent in sheep, where nutritional changes such as fasting and glucose infusion cause a smaller change in plasma IGFBP-2 concentrations compared to that of plasma IGFBP-1, particularly in fetal lambs (Osborn *et al.* 1992). These results are consistent with a recent report that insulin treatment of diabetic rats normalizes hepatic IGFBP-1 mRNA within 1 h after treatment, whereas such a response in IGFBP-2 mRNA does not occur even 6 h after injection (Ooi *et al.* 1992). Ooi *et al.* (1992) also noted that increases in hepatic IGFBP-2 mRNA were not accompanied by corresponding changes at gene transcription levels, indicating that the increase in steady state levels of IGFBP-2, unlike IGFBP-1, is a post-transcriptional event. These findings indicate that regulation of IGFBP-2 may be different from that of IGFBP-1, despite their similar responses to metabolic changes, and further suggest that IGFBP-2 may provide more chronic adaptation to metabolic change than IGFBP-1. Such a different regulation of IGFBP-2 is further expressed by its inverse relationship to GH status. In humans, serum IGFBP-2 levels significantly increase in GH-deficient patients, such as in hypopituitarism (Hardouin *et al.* 1989), whereas the concentrations markedly decrease in normal human

subjects treated with GH or patients with extrapancreatic tumour hypoglycaemia (Zapf *et al.* 1990). Similarly, in sheep with different physiological status (Hodgkinson *et al.* 1991*a*; Davis *et al.* 1992; Gallaher *et al.* 1995) and lactating dairy cows (Cohick *et al.* 1992; Sharma *et al.* 1994), GH treatment markedly suppresses circulating concentrations of IGFBP-2. However, GH does not affect serum IGFBP-2 levels in calorically restricted adults (Clemmons *et al.* 1991; Young *et al.* 1992). Consistent with these reports, GH is unable to suppresses hepatic IGFBP-2 mRNA in hypophysectomized rats (Gosteli-Peter *et al.* 1994). Since circulating concentrations of insulin are suppressed in these studies, these data suggest that the presence of adequate levels of insulin may be required for GH to suppress plasma IGFBP-2 levels.

While IGFBP-1 and IGFBP-2 may provide short- and medium-term adaptation to metabolic change, respectively, IGFBP-3 seems to be involved in long-term adaptation to metabolic variation. This is reflected by the smaller changes in plasma IGFBP-3 levels observed during a 14-day diet restriction compared with IGFBP-1 and IGFBP-2 in human subjects (Young *et al.* 1992). A similar situation is also evident in lactating cows in which complete feed deprivation for 48 h failed to alter circulating concentrations of IGFBP-3 while affecting plasma concentrations of IGFBP-2 (McGuire *et al.* 1995). Furthermore, IGFBP-3 responds to a metabolic change in a manner virtually opposite to IGFBP-1 and IGFBP-2. Thus, circulating concentrations of IGFBP-3 are reduced by starvation and markedly increased with refeeding in rats (Zapf *et al.* 1989), pigs (McCusker *et al.* 1989) and lactating cows (McGuire *et al.* 1995). As a result, these metabolic changes lead to an inverse relationship between circulating concentrations of IGFBP-3 and IGFBP-2. Likewise, GH treatment markedly increases circulating concentrations of IGFBP-3, while concurrently decreasing plasma IGFBP-2 levels in several species, including sheep (Hodgkinson *et al.* 1991*a*; Davis *et al.* 1992), dairy cows (Cohick *et al.* 1992; Sharma *et al.* 1994; McGuire *et al.* 1995) and pigs (Evock *et al.* 1990; Coleman & Etherton, 1991). Such a constant inverse relationship between IGFBP-3 and IGFBP-2 suggests that IGFBP-3 and -2 may be regulated in a coordinated manner although the mechanism involved remains unclear. This suggestion is further supported by the fact that developmental changes in plasma IGFBP-2 and -3 concentrations are regulated inversely in a number of species including sheep (Gallaher *et al.* 1992; Carr *et al.* 1995), cattle (Skaar *et al.* 1994) and pigs (Lee *et al.* 1992;

McCusker *et al.* 1991; Owens *et al.* 1991). For example, in sheep, plasma levels of IGFBP-2 and IGFBP-2 mRNA in the liver are high in the fetus in mid to late gestation and decrease towards adult levels near term, whereas the reverse is true for IGFBP-3 over the same period (Carr *et al.* 1995). In ruminants such as sheep and cattle, a major increase in plasma IGFBP-3 concentrations in postnatal life coincides with the appearance of the hepatic GHR (Butler & Gluckman, 1986), suggesting that hepatic GHR may be involved in regulating plasma IGFBP-2 and -3 concentrations. This suggestion is consistent with the abovementioned reports that circulating concentrations of plasma IGFBP-2 and -3 are sensitive to nutritional and GH status, which are important components in up-regulating hepatic GHR in ruminants. However, an increase in plasma IGFBP-3 occurs even in GH-deficient humans (Kanety *et al.* 1993) and rats (Clemmons *et al.* 1989; Zapf *et al.* 1989), when they are treated with IGF-I. For example, in patients with Laron-type dwarfism (LTD) which are characterized by the absence of GH receptor activity, chronic treatment with IGF-I increases serum IGFBP-3 up to 19-fold after six months of therapy (Kanety *et al.* 1993). These data indicate that GHR may not be an essential component in regulating plasma IGFBP-3 levels and further suggest that plasma IGF-I may be crucial in regulating IGFBP-3. Further support is given in recent reports with sheep (Carr *et al.* 1995) and cattle (Skaar *et al.* 1994), where developmental changes in plasma IGFBP-3 were studied, and in which its concentration was correlated positively with circulating concentrations of IGF-I. In one of these studies, where circulating concentrations of IGFBP-2 and IGF-II were measured simultaneously (Carr *et al.* 1995), a developmental change in plasma IGFBP-2 was correlated positively with changes in plasma IGF-II, indicating a similar mechanism is operating between IGF-II and IGFBP-2.

The physiological significance of the IGFBPs still remains unclear. Earlier studies with IGFBPs have shown that soluble IGFBPs inhibit mitogenic activities of IGFs *in vitro* (Cohick & Clemmons, 1993; Etherton, 1993), suggesting that IGFBPs directly modulate the action of IGFs at the target cells. These findings, together with the fact that IGFBPs increase the half-life of IGF-I and -II (Hodgkinson *et al.* 1987; Davis *et al.* 1989*b*; Ballard *et al.* 1991), have led to a suggestion that IGFBPs act as a reservoir of IGFs. This may be particularly true with regard to IGFBP-3 since IGF-I or -II in association with this binding protein have greatly extended half-lives. For example, in

sheep, the half-life of IGF-I is greatly increased from less than 10 min when IGF-I is not bound to any IGFBPs, to 545 min when it is bound to IGFBP-3 (Davis *et al.* 1989*b*). In contrast, mutants of IGF-I including des(1-3) IGF-I, which have a markedly reduced affinity for IGFBPs, are cleared more rapidly (Cascieri *et al.* 1988; Ballard *et al.* 1991). Given that serum contains high levels of IGFs and that any free IGFs beyond the binding capacity of the IGFBPs induce hypoglycaemia, such a capture of IGFs by IGFBP-3 with the gradual release of IGFs into target tissues is physiologically important. In sheep (Davis *et al.* 1992) and cows (Cohick *et al.* 1992), GH treatment increases IGFBP-3 concentrations in plasma with no corresponding changes in afferent mammary lymph. In contrast, exogenous GH simultaneously decreased IGFBP-2 levels in both plasma and mammary lymph. These observations suggest that IGFBP-3, but not IGFBP-2, does not leave the circulation. This further emphasizes the role of IGFBP-3 as a reservoir of IGFs.

While IGFBP-3 may function mainly as a reservoir of IGFs, smaller forms of IGFBPs may be involved in mediating transport of the IGFs from the vascular space to target cells. This is particularly true with regard to IGFBP-1 and -2 since both binding proteins have been shown to cross intact vascular endothelium in rats (Bar *et al.* 1990*a*,*b*), sheep (Hodgkinson *et al.* 1991*b*; Davis *et al.* 1992) and cattle (Cohick *et al.* 1992). For example, ^{125}I-IGFBP-1 and -2, when perfused through the isolated rat heart, cross the capillary boundaries of the cardiac microcirculation and distribute in subendothelial tissues (Bar *et al.* 1990*a*). However, it is unclear whether the IGFBP complexes are translocated into the extravascular space in an intact form or whether dissociation occurs, enabling transfer of the free components with subsequent reassembly of the complexes.

While earlier studies have shown that IGFBPs inhibit biological actions of IGFs, a recent study has reported that purified IGFBP-3 can potentiate biological actions of IGF-I under certain conditions (Conover *et al.* 1990). In that study, using a bovine fibroblast model, a bIGFBP-3 purified from bovine serum was either coincubated or preincubated with IGF-I and [^{3}H]aminoisobutyric acid (AIB) uptake measured. Results showed that coincubation of IGF-I with increasing concentrations of IGFBP-3 produced a dose-dependent inhibition of IGF-I-stimulated [^{3}H]AIB uptake in cultured bovine fibroblasts. This inhibition was complete at equimolar concentrations of IGF-I and bIGFBP-3. In

contrast, preincubation with bIGFBP-3 resulted in a dose-dependent enhancement of IGF-I-stimulated [^{3}H]AIB uptake; IGF-I bioactivity was increased by 32-86% after a 24 h pre-exposure to bIGFBP-3 and a 2- and 6-fold increase was observed after a 72 h preincubation. These observations suggest that the inhibitory effects of IGFBPs on IGFs actions found in the earlier studies are associated with a short period of incubation with IGFBPs. In this situation, IGFBPs inhibit the biological action of IGFs by sequestering and preventing IGF-I receptor binding since inhibition of IGF-I-stimulated [^{3}H]AIB uptake parallels the ability of bIGFBP-3 to prevent [^{125}I]IGF-I cell surface binding (Conover *et al.* 1990). In contrast, the potentiating effects of bIGFBP-3 are associated with increased binding of IGF-I to membrane-associated bIGFBP-3. In a follow-up study with bovine fibroblasts, Conover (1992) reported that cell-associated IGFBP-3 gradually loses its affinity for IGF-I as the incubation time progresses. As a result, it has a 10-fold lower affinity for IGF-I compared to intact IGFBP-3 in solution after 72 h of exposure to bovine fibroblasts. Consistent with this, McCusker *et al.* (1990) reported that IGFBPs on the surface of human fibroblasts have lower affinity for IGF-I than do IGFBPs in solution. Therefore, the potentiating effect of cell surface-associated IGFBP-3 may be due to a progressive reduction in its binding affinity. This suggestion from *in vitro* studies is further supported by a recent *in vivo* study (Stewart *et al.* 1993) in which systemic administration of anti-IGF-I serum, which has been pre-incubated with IGF-I, significantly stimulated daily gains of dwarf mice relative to those of animals treated with IGF-I alone at the same dose or even a 2.5-fold higher dose. Such a gradual release of IGF-I in the vicinity of its receptor through a progressive reduction in affinity appears to be important in producing an optimal biological response since IGFBP-3 blocks IGF-I-induced receptor down-regulation and cell desensitization in cultured bovine fibroblasts (Conover & Powell, 1991).

1.2.6 IGF receptors

Two distinct, but obviously related, receptors for IGF-I and IGF-II have been identified (Kasuga *et al.* 1981; Massague & Czech, 1982). These peptides have been designated IGF-I and IGF-II receptors. The presence of these receptors has been demonstrated in a wide variety of tissues.

The IGF-I receptor binds IGF-I with higher affinity than IGF-II and also binds

insulin (Massague & Czech, 1982). It consists of three disulfide-linked forms structurally similar to the insulin receptor. Both peptides have two αß subunits, which are linked to each other by the disulfide bond to form the active receptor (Massague & Czech, 1982). The α subunits contain binding sites, whereas ß subunits contain tyrosine kinase (Massague & Czech, 1982). Given this structural and functional similarity, it is not surprising that IGF-I receptors bind insulin as well as IGF-I and IGF-II. However, insulin interacts with the IGF-I receptor only at supraphysiological concentrations because of its low affinity for that receptor. Furthermore, circulating concentrations of IGF-I are much higher than those of insulin. Therefore, it is unlikely that insulin binds to IGF-I receptors in normal physiological conditions. This specificity of binding is critical for the maintenance of the specificity of biological actions of each peptide.

The presence of tyrosine kinase in the two ß subunits of the IGF-I receptor suggests that a multiple transmembrane signalling, leading to the final characteristic actions of IGFs, may be initiated through these ß subunits. *In vitro*, IGF-I has been shown to stimulate tyrosine-specific phosphorylation of the ß subunits of the IGF-I receptor (Rubin *et al.* 1983). This *in vitro* result is confirmed by an *in vivo* study in which tyrosine-specific phosphorylation of the ß subunits of the IGF-I receptor is enhanced in a dose-dependent manner in the presence of IGF-I (Jacobs *et al.* 1983). In both studies, insulin was also effective in activating phosphorylation, although this action required a much higher dose of insulin. The most important phosphorylation occurs on tyrosine residues at positions 1146, 1150 and 1151 of the insulin receptor since simultaneous mutation of these regions results in a biologically inactive receptor (Murakami & Rosen, 1991). However, there is some evidence that, in addition to these autophosphorylation sites of the ß subunit, the tyrosine residue at position 960 is required for full biological activity of the insulin receptor (White *et al.* 1988). A similar mechanism appears to be operative in the IGF-I receptor since the tyrosine residue at position 950 is required for IGF-I signal transduction in the pituitary somatotroph (Yamasaki *et al.* 1992).

The IGF-II receptor, on the other hand, is structurally and functionally quite different from the IGF-I and insulin receptors. It is composed of a monomeric protein (M_r 258,000-268,000) which is not disulfide-linked to any other membrane component (Massague & Czech, 1982). It has a higher affinity for IGF-II than for IGF-I, and has no significant affinity for insulin (Kasuga *et al.* 1981; Massague & Czech, 1982).

Likewise, it does not have intrinsic tyrosine kinase activity (Corvera *et al.* 1986). Instead, the IGF-II receptor has been shown to be identical to the cation-independent mannose-6 phosphate (M-6-P) receptor (MacDonald *et al.* 1988; Oshima *et al.* 1988). These observations are further confirmed by biochemical and immunological studies (Kiess *et al.* 1988; Tong *et al.* 1988). The M-6-P is thought to be important in the intracellular targeting of enzymes to lysosomes. In addition, the M-6-P binding to the receptor enhances its receptor affinity for IGF-II (Roth *et al.* 1987; Tandeski *et al.* 1990). The physiological significance of this enhanced binding is unclear, although the close linkage of the M-6-P/IGF-II receptor to the lysosomal system suggests that the M-6-P/IGF-II receptors may be involved in intracellular degradation of bound IGF-II. However, in ovine hepatic tissues where ^{125}I-hIGF-II binds specifically to the IGF-II receptor, treatment of the cells with trypsin or sodium acetate fails to remove a significant proportion of the surface-bound IGF-II (Tandeski *et al.* 1990). Furthermore, lysosomal inhibitors such as ammonium chloride, chloroquine and leupeptin do not affect ^{125}I-hIGF-II degradation or cell associated radioactivity (Tandeski *et al.* 1990). These data suggest that a significant fraction of the receptor-bound IGF-II is internalized but not degraded by these cells. Therefore, the IGF-II receptor may not function as a degradative pathway for IGF-II, at least in the sheep hepatocyte.

The IGF-II/M-6-P receptor can be cleaved from the cell surface, resulting in the release of a truncated form of the IGF-II/M-6-P receptor (200-250 kDa) into the circulation. In fact, there is considerable evidence that this truncated form of the IGF-II receptor functions as an important IGF-II binding protein in the circulation of a variety of species, including sheep (Hey *et al.* 1987; Gelato *et al.* 1989; Lord *et al.* 1991; Gallaher *et al.* 1992, 1994). For example, in fetal lambs, approximately 50 % of circulating IGF-II is associated with the IGF-II/M-6-P receptor fragment (Gelato *et al.* 1989). However, the circulating IGF-II /M-6-P receptor is markedly reduced in post-natal life, carrying less than 7 % of the circulating IGF-II (Gelato *et al.* 1989). A similar developmental change in the IGF-II/M-6-P receptor has been reported in rats (Kiess *et al.* 1987*a*; Bobek *et al.* 1992) and humans (Funk *et al.* 1992), although the degree of developmental regulation of this protein is much less in the latter case. The mechanism which regulates this protein remains largely unknown. A recent study with fetal lambs has shown that fasting reduces circulating levels of the IGF-II/M-6-P receptor, whereas

glucose and insulin infusion reverse this trend (Gallaher *et al.* 1994). These data suggest that nutritional status may play an important role in regulating the IGF-II/M-6-P receptor.

The role of the IGF-II receptor in mediating IGFs-induced growth has not been established. Although Tandeski *et al.* (1990) suggested that the mitogenic actions of IGF-II may be exerted by an intracellular pathway via the hepatic IGF-II receptor, a large body of evidence with antireceptor antibodies has concluded that such effects are mediated solely by the IGF-I receptor. For example, Kiess *et al.* (1987*b*) reported that both IGF-I and IGF-II mediate their growth promoting effects through the IGF-I receptor and not through binding to the IGF-II receptor. However, the antireceptor antibodies do not always completely inhibit binding nor are they necessarily specific inhibition of binding (Kojima *et al.* 1988; Steele-Perkins & Roth, 1990) or functional as IGF-I or IGF-II receptor antagonists (Kojima *et al.* 1988; Steele-Perkins *et al.* 1988). Recently, an IGF-II analogue that selectively interacts with the IGF-II receptor with normal affinity, but with markedly reduced affinities for the IGF-I and insulin receptors, has been developed (Beukers *et al.* 1991). This provides new ways to identify which actions of IGF-II are mediated via the IGF-II receptor and which are due to cross reactivity with the IGF-I and insulin receptors. Rosenthal *et al.* (1994) were probably the first group to study the role of the IGF-II receptor in IGFs-induced cell growth with this type of analogue ([Leu^{24}]IGF-II). Results have shown that this IGF-II analogue is able to stimulate muscle cell differentiation (BC3H-1) at concentrations that do not significantly interact with IGF-I receptor. This finding is the first evidence of a direct role of the IGF-II receptor in IGF-induced myogenesis and questions the values of previous reports associated with receptor antibodies. However, further studies may be required before definitive conclusions can be made with regard to the role of IGF-II receptors in cell growth.

1.2.7 Endocrine and paracrine/autocrine functions of IGF-I

Since the first report that IGF-I is synthesized and secreted by a number of tissues (D'Ercole *et al.* 1984), there have been many studies showing that almost every tissue examined contains IGFs and their mRNA (Cohick & Clemmons, 1993; Etherton, 1993). Furthermore, most of these tissues also synthesize and secrete IGFBPs. These findings

strongly suggest that paracrine/autocrine actions of IGFs may be critical in any observed biological action of GH.

Direct evidence for paracrine/autocrine actions of IGFs has been provided by immunoinhibition studies. Under *in vitro* conditions the addition of anti-IGF antibodies or antireceptor antibodies blocks DNA synthesis in human fibroblasts and porcine smooth muscle cells that secrete these peptides (Clemmons & Van Wyk, 1985). Similarly, regional infusion of IGF-I antibodies into a perfused hindlimb from rats has been shown to block both IGF-I and GH-stimulated growth (Schlechter *et al.* 1986). These *in vitro* findings are consistent with an *in vivo* study (Palmer *et al.* 1993) in which administration of GH significantly stimulated growth in normal growing rats while having no effects on circulating concentrations of IGF-I. A similar finding has also been reported in hypophysectomized rats (Orlowski & Chernausek, 1988) in which systemic administration of GH stimulated growth and increased tissue IGF-I levels without corresponding increases in circulating IGF-I. These data, together with a recent report that immunization of growing guinea pigs with an anti-IGF-I monoclonal antibody failed to alter growth (Kerr *et al.* 1990), suggest that an autocrine or paracrine action of IGF-I plays a critical role in regulating growth of animals.

Although the above evidence emphasizes the importance of locally produced IGF-I in regulating growth, this does not necessarily exclude the possibility of an endocrine role of IGFs. As discussed previously, IGFBPs, particularly with smaller molecular forms, can cross the vascular barrier, thus serving their endocrine role. Consistent with this, a large body of evidence has shown that systemically administered IGF-I stimulates growth in GH-deficient animals as well as normal animals, although the potency of IGF-I is much lower than that of GH (Robinson & Clark, 1989). Koea *et al.* (1992) have also shown that passive immunization against circulating IGF-I increases protein metabolism in growing lambs, demonstrating a physiological role of circulating IGF-I. Further direct evidence for an endocrine role of IGF-I in growth has been provided by Pell *et al.* (1993) who studied regulation of transcription initiation from IGF-I leader exons and of tissue IGF-I expression in response to changed GH and nutritional status in growing lambs. Results have shown that in conditions of maximal growth, the increase in circulating concentrations of IGF-I correlated with an increased content of exon 2 transcripts in liver IGF-I mRNA. In contrast, in peripheral tissues such as muscle, only

exon 1 IGF-I transcripts predominate and they remain at constant levels across a broad range of nutritional status and GH levels. These data suggest that IGF-I-stimulated skeletal growth is controlled principally by the liver, which acts as a sensor of anabolic status and which signals growth potential to target tissues via increased output of circulating IGF-I.

In conclusion, the above data suggest that the growth-promoting and anabolic actions of GH are mediated in both an endocrine and an autocrine/paracrine manner, although the relative importance of each mode of action remains unclear.

1.3 BIOLOGICAL ACTIONS OF GROWTH HORMONE (GH)

The potential of GH to enhance farm animal production has been extensively assessed in a number of areas. These include foetal growth, mammary development, postnatal growth and milk production. Therefore, these areas will be discussed in the following sections.

1.3.1 Fetal development and maternal metabolism

It is well recognized that fetal concentrations of GH in several species, including sheep, are elevated compared to adult values (see section **1.2.1 Nature and secretion of growth hormone**). For example, plasma GH concentrations in fetal lambs and calves were 10 to 20 times greater than those of their dams during the last third of pregnancy (Bassett *et al.* 1970; Reynolds *et al.* 1990). These elevated levels of GH in the fetus appear to be due to the immature state of the hypothalamic-pituitary axis (de Zegher *et al.* 1989*a,b*; Silverman *et al.* 1989). This suggestion is supported by the observation that IGF levels in mid-gestation ovine fetuses are not affected by decapitation or electronic destruction of the fetal medial-basal hypothalamus, which abolishes GH secretion (Gluckman & Butler, 1985). Therefore, the reduction of foetal GH concentrations by fetal hypophysectomy, hypophyseal stalk section or decapitation has little effect on fetal growth (Gluckman & Liggins, 1984; Parkes & Hill, 1985).

The inability of GH to regulate fetal growth may be due to a lack of GHRs in fetal tissues (Gluckman *et al.* 1983; Freemark *et al.* 1986, 1988; Klempt *et al.* 1993). It is well recognized that the appearance of the GHR postnatally is crucial for IGF-I production and thus GH-dependent growth (see section **1.2.2 Growth hormone**

receptors (GHR)). Although GHR are present as early as day 51 of gestation in hepatic tissue of the ovine fetus, a recent study has shown that oGHR mRNA present at this stage differs structurally from the transcript present in late gestation fetal liver and adult liver (Pratt & Anthony, 1995). These data suggest that the GHR of the fetus may be non-functional and provides a further explanation for earlier observations that fetal GH has no effects on prenatal growth of lambs (Gluckman & Liggins, 1984; Parkes & Hill, 1985).

Fetal development is critically dependent upon an adequate nutrient supply across the placenta which, in turn, is largely dependent on maternal nutrition and metabolism (Owens, 1991). For example, maternal fasting for 3 days during late gestation causes fetal growth retardation in the rat (Davenport *et al.* 1990). It is well recognized that GH has a role in coordinating nutrient partitioning to target tissues under various physiological status. For example, GH directs nutrient partitioning to the mammary gland during lactation (see section **1.3.4 Lactation**). This raises the possibility that exogenous GH in dams might stimulate foetal development by altering maternal nutrient partitioning in favour of fetal growth.

Such a hypothesis has been tested in several species including sheep (Kann *et al.* 1989), cattle (Stelwagen *et al.* 1992; Lucy *et al.* 1995), pigs (Kelley *et al.* 1995; Sterle *et al.* 1995) and rats (Chiang & Nicoll, 1991; Gargosky *et al.* 1991). However, there appear to be conflicting results among these studies. While in some studies administration of GH to pregnant dams stimulated fetal growth (Kann *et al.* 1989; Kelley *et al.* 1995; Sterle *et al.* 1995), in others it did not (Chiang & Nicoll, 1991; Gargosky *et al.* 1991; Stelwagen *et al.* 1992). Indeed, in one of these studies (Chiang & Nicoll, 1991), relatively large doses (5 mg/d) of GH administered to the pregnant rat decreased fetal weights or even cause an advanced stage of fetal and placental reabsorption.

The reasons for these conflicting results among studies remain unclear, but may be related to the stage of gestation when GH treatment commences. In the majority of studies where maternal treatment with GH stimulated fetal growth, GH treatment commenced in the early stages of gestation (Kelley *et al.* 1995; Sterle *et al.* 1995). In contrast, in the majority of studies where maternal treatment with GH was unable to stimulate foetal growth (Chiang & Nicoll, 1991; Gargosky *et al.* 1991), GH treatment

started in the second half of gestation. It is recognized that pregnant animals actively deposit body fat during the early stage of gestation, which is later mobilized to support the increasing nutrient demands of the fetus. Therefore, GH treatment in early gestation, as opposed to later in gestation, may divert nutrients from dams to placenta and thus influence implantation and placental growth by reducing the limitation of placental size on fetal growth. Indeed Sterle *et al.* (1995) reported that treatment of pregnant gilts with pGH (5 mg/d) from days 30 to 43 of gestation increased placental and fetal weights when gilts were killed on day 44 of gestation. Further analysis of the relationship between fetal weight and implantation lengths (of the vascular area of the uterus) showed that exogenous GH had the greatest effects on the fetuses in the shortest implantation class (based on implantation lengths). This pronounced effect of GH treatment on growth in the fetus with shorter implantation lengths suggests that GH corrects for a limiting factor, related to small placental contact area, by enhancing nutrient transfer across the diffuse placenta and thus uptake of nutrients by the fetus. Such an enhanced nutrient supply to the fetus may result in increased fetal plasma levels of IGF-I which stimulate fetal growth.

On the other hand, the inability of GH to affect fetal development during later stages of gestation may be related to the development of maternal GH resistance at this stage. Such a development of GH resistance in the later stage of gestation is associated with a developing catabolic state which leads to a reduction in plasma concentrations of IGF-I via the down-regulation of GHR (see section **1.2.2 Growth hormone receptors (GHR)**). In fact, there is some indication that these low plasma levels of IGF-I are responsible for the lack of anabolic effects of GH observed in the late gestation period. In the studies (Chiang *et al.* 1990; Chiang & Nicoll, 1991; Gargosky *et al.* 1991), where maternal treatment with GH had no anabolic effect, maternal circulating concentrations of IGF-I were not affected. In contrast, in a situation where maternal GH treatment alters maternal circulating concentrations of IGF-I, GH treatment shows anabolic actions. For example, Chiang *et al.* (1990) demonstrated that administration of a high dose of bGH (10 mg/kg/d) to the pregnant rat overcame GH resistance and fully restored fetal paw growth which was reduced by 40% in the second stage of gestation. In that study, effectiveness of treatment was correlated with the restoration of maternal IGF-I concentrations, indicating that growth response was mediated by plasma concentrations

of IGF-I. This suggestion is further supported by a more recent study (Gargosky *et al.* 1991) in which infusion of IGF-I to the late-pregnant rat increased maternal weight gain. Such an anabolic action occurred without changes in fetal or placental growth.

Restoration of maternal anabolic actions of GH in the later stage of gestation suggests that GH treatment may induce nutrient partitioning to the dam in favour of maternal growth instead of fetal growth. This modification in the mode of maternal nutrient repartitioning during late gestation could lead to a detrimental effect on fetal growth. Such a possibility was examined directly by Chiang and Nicoll (1991). In that study, pregnant rats fed a reduced diet (60% of *ad libitum*) were treated with GH during day 10-20 of gestation. Maternal inguinal fat store and tibial epiphyseal plate width (TEPW), maternal plasma IGF-I concentrations, placental and fetal weights were compared to those in the saline-treated group. A restricted diet reduced inguinal fat stores, depressed IGF-I concentrations and reduced TEPW relative to those of *ad libitum*-fed animals, but did not affect the growth of the fetus or placenta. In contrast, treatment of dams with GH (1 mg or 5mg/d) increased fat stores, IGF-I concentrations and TEPW growth but reduced fetal and placental weights. Dams treated with the high dose of GH were in an advanced stage of fetal and placental reabsorption by day 20 of gestation. Consistent with this, Kvergas *et al.* (1986) reported that GH treatment of pregnant pigs during late gestation resulted in a 20% death rate of neonates, with most of the deaths coming within hours of parturition. These data strongly indicate that maternal resistance to the anabolic actions of GH is an important adaptation for diverting nutrients from the mother to the fetus.

In conclusion, the above data suggest that exogenous GH may have a potential in the manipulation of fetal development. However, the timing of such an exploitation appears to be critical since GH treatment at the later stages of gestation could have a detrimental effect on fetal growth.

1.3.2 Postnatal growth

While GH treatment has little effect on prenatal growth, it has been consistently shown to have stimulatory effects on postnatal growth. In growing pigs, pGH treatment usually increases average daily gains (ADG) by 10 to 20 % (Kopchick & Cioffi, 1991). This is accompanied by a concomitant decrease in feed intake. The net result of these

effects is a marked improvement in feed efficiency by up to 35% (Kopchick & Cioffi, 1991). pGH treatment also improves carcass characteristics of the pig, most notably through a large reduction in fat deposition (Kopchick & Cioffi, 1991).

The reduced feed intake observed in pigs treated with pGH is interesting since such a decrease in feed intake occurs even during late gestation and lactation when sows are treated with recombinant pGH (rpGH) (Cromwell *et al.* 1992). In contrast, rpGH treatment increases feed intake in the rat (Azain *et al.* 1995), suggesting that the effect on feed intake is species-specific. It has been suggested that the reduced feed intake observed in the pGH-treated pig is due to a decrease in net energy deposition (Azain *et al.* 1995). This decrease in net energy deposition is directly related to the composition of the gain in this species, in which pGH markedly decreases lipid deposition while causing a small increase in protein deposition (Campbell *et al.* 1990). This situation in the pig is in contrast to that in the rat, where pGH increases total energy deposition by markedly increasing protein deposition with a small decrease in fat accretion (Azain *et al.* 1995). The different responses in carcass composition and feed intake to exogenous GH between species emphasise the different feeding strategies needed to optimize the response to GH in each situation. For example, in the pig, where exogenous GH has a small effect on protein deposition and reduces feed intake, a greater concentration of dietary protein may be required to optimize the response to pGH, as suggested previously (Campbell *et al.* 1990, 1991*b*).

A similar but more equivocal situation exists for ruminants. While some studies have shown that GH treatment enhances growth rate and feed efficiency in sheep and cattle (Johnsson *et al.* 1985; Beermann *et al.* 1990; Enright *et al.* 1990; Groenewegen *et al.* 1990; Pell *et al.* 1990; McLaughlin *et al.* 1991, 1993*a*,*b*, 1994; Preston *et al.* 1995), others have not (Muir *et al.* 1983; Olivares *et al.* 1990; Murphy *et al.* 1991; Rosemberg *et al.* 1989; Neathery *et al.* 1991; Sun *et al.* 1992; Schwarz *et al.* 1993). These conflicting results among studies may be partly associated with differences in the mode of GH administration. For example, in the majority of studies in which GH failed to have growth-promoting effects, the GH was administered at two day (Olivares *et al.* 1990) or longer (Murphy *et al.* 1991; Schwarz *et al.* 1993) intervals. The inability of GH to stimulate growth in these conditions may be associated with changes in hepatic GHR since single bolus injections of GH acutely down-regulate the number of GH receptors

(Maiter *et al.* 1988*b*). This down-regulation of hepatic GHR may also provide a possible explanation for a recent report that daily injection of GH as high as 0.3 mg/kg LW/day had negative effects on growth parameters, including growth rate, in finishing beef steers (Moseley *et al.* 1992). Recently, Azain *et al.* (1995) compared the effects of daily versus continuous administration of pGH on growth rate in intact female rats using four different doses (0, 0.4, 1.2, and 3.6 mg of pGH/d). Results showed that there was a dose-dependent increase in rate of gain with an interaction of dose and mode of delivery. At the low dose (0.4 mg/day) daily injection of pGH stimulated growth, whereas such a response was absent with continuous administration of pGH. Conversely, at the high dose (3.6 mg/d) continuous delivery was more efficacious than daily injection in stimulating growth. These data suggest that dose of GH may be crucial in choosing which mode of GH administration is most effective.

In studies in which animals are treated with GH for periods of up to 8 weeks (Lemal *et al.* 1989; Beermann *et al.* 1990; McLaughlin *et al.* 1991, 1993*a*,*b*, 1994), GH administration usually results in a 10 to 20% increase in (ADG). In general, such a response occurs without a concomitant increase in voluntary feed intake. Thus, the increased feed conversion rate (FCR) observed in ruminants treated with GH, unlike that reported in the pig, is solely a function of an increased growth rate. However, in some of these studies, such responses are not apparent until several weeks after GH treatment. For example, in growing lambs (Beermann *et al.* 1990) and heifers (Lemal *et al.* 1989), stimulatory effects of exogenous GH on growth were not detected until 4 weeks after GH treatment commenced. These data indicate that a minimum of 4 weeks of treatment is required for significant effects of GH to be achieved in growing ruminants. Furthermore, these results may provide a possible explanation for the lack of growth response observed in the earlier studies (Muir *et al.* 1983; Rosemberg *et al.* 1989) since GH treatment lasted only for 4 weeks in those studies. An enhanced growth response has been reported in the longer-term studies (Johnsson *et al.* 1985; Enright *et al.* 1990; Groenewegen *et al.* 1990; Pell *et al.* 1990; Preston *et al.* 1995) where animals were treated with GH for a minimum period of 12 weeks. For example, Pell *et al.* (1990) reported that daily administration of GH (0.1 mg/kg LW/day) over a period of 12 weeks enhanced daily growth rate by 36% in lambs.

In most studies with ruminants, GH treatment had less effect on carcass weights

than on liveweights. These results suggest that the increased live weight gains observed during GH treatment are due primarily to an increase in non-carcass components of the body such as the head, blood, gut, greasy fleece, feet and major organs, as reported previously (Johnsson *et al.* 1985; Zainur *et al.* 1989; Pell *et al.* 1990). However, in a situation where relatively large doses of GH are used, GH treatment sometimes leads to a significant increase in carcass weight. For example, Sandles and Peel (1987) reported that administration of relatively large doses of bGH (0.6 mg/kg LW/d) to growing heifers resulted in a 9% increase in carcass weight.

While GH treatment has only a limited effect on carcass weight, it has a marked influence on the composition of the carcass, especially the fat and protein content. The relative changes of these components appear to be dependent on stage of growth when GH treatment commences. In general, in young growing ruminants where growth is mainly associated with a greater proportion of protein deposition (Fourie *et al.* 1970; Searle *et al.* 1972; Berg & Butterfield, 1976), GH has a greater effect on protein content rather than on fat content (Johnsson *et al.* 1985; Rosemberg *et al.* 1989; Beermann *et al.* 1990; Pell *et al.* 1990). In contrast, in finishing sheep and cattle where growth is largely associated with a greater proportion of fat deposition (Fourie *et al.* 1970; Searle *et al.* 1972; Berg & Butterfield, 1976), GH treatment has a greater effect on fat content than on protein content (Moseley *et al.* 1992; Schwarz *et al.* 1993; McLaughlin *et al.* 1993*a*, 1994; Preston *et al.* 1995). In both situations, the net result is that GH-treated ruminants contain a greater proportion of lean tissue with a concomitant reduction of fat content than those in untreated animals.

It is generally accepted that, in growing animals, the decreased body fat content associated with GH treatment is primarily due to decreased lipogenesis rather than to increased lipolysis. Similarly, the increased muscle mass appears to be due solely to increased protein synthesis with little or no change in degradation rates. This is particularly true with regard to ruminants. Increased nitrogen retention and whole body protein synthesis have been reported in beef heifers treated with GH (Eismann *et al.* 1986*a*), but there was no change in protein catabolism. Similar results have also been reported in sheep (Pell & Bates, 1987). In a more recent study with steers (Eismann *et al.* 1989) bGH increased the incorporation of [^{14}C]leucine into protein in all tissues examined while reducing the whole-body leucine oxidation. A similar result has been

observed in the pig (Caperna *et al.* 1995) in which pGH treatment resulted in a 67% increase in the average deposition rate of each amino acid. As a result of this effect on protein synthesis, plasma concentrations of most of the essential amino acids, including leucine, were decreased in GH-treated steers (Preston *et al.* 1995).

Although a growth response in young ruminants has been reported at doses as high as 0.6 mg/kg LW/day (Sandles & Peel, 1987), there is some evidence that the optimum dose of GH for maximum FCR is lower than that required for maximal growth rate (Moseley *et al.* 1992; McLaughlin *et al.* 1994). This is particularly true with finishing animals. Moseley *et al.* (1992) conducted two major experiments to determine the efficacy of various doses of rbGH on ADG, FCR and carcass composition in finishing beef steers. In the first experiment, crossbred beef steers received daily intramuscular (i.m.) injections of 33, 100 and 300 ug/kg LW per day of GH, whereas in the second experiment, animals received daily i.m. injections of 8.25, 16.5, 33, or 66 ug/kg of LW of rbGH. ADG changed linearly with increasing dose in the first experiment, whereas FCR responses were quadratic, with the maximum response occurring at 33 ug/kg LW/day. In experiment 2, steers receiving 16.5 and 33 ug/kg GH responded with improved ADG and FCR, whereas steers receiving 8.25 and 66 ug/kg GH responded with improved FCR but not ADG relative to the saline-treated group. These results indicate that a daily dose between 16.5 and 33 ug/kg LW/day is required to optimize both ADG and FCR in finishing beef steers. A similar result has been reported in finishing lambs (41 kg) (McLaughlin *et al.* 1994) in which ADG and FCR peaked at dose of 50 mg/week with both traits being reduced at the higher dose (75 mg/week). However, in both studies, GH effect on carcass composition were apparent in a dose-dependent manner up to the highest doses. These data suggest that the optimum dose of GH may vary depending on the traits of economic importance.

The changes in the growth rate and carcass composition of the GH-treated ruminant suggests that modifications in the animals' diet, such as inclusion of greater concentrations of dietary protein (as suggested for the pig), may be required to adequately support the optimal growth response to GH in growing sheep and cattle. This may be particularly true with young growing animals, where growth is largely associated with protein rather than fat deposition. In fact, Beermann *et al.* (1991) reported that abomasal casein infusion further enhanced nitrogen retention by 43% when lambs were

injected twice daily with rbGH (0.2 mg/kg LW/d) for 15 days. A similar result has also been observed in growing steers (Houseknecht *et al.* 1992). These data suggest that the growth response to exogenous GH may be limited by quantity and(or) quality of amino acids in young animals. In association with this, it is worthwhile noting that chronic treatment with GH sometimes leads to significant increases in voluntary feed intake of young lambs (Pell *et al.* 1990) and calves (Groenewegen *et al.* 1990). For example, Pell *et al.* (1990) reported that a chronic treatment with rbGH over a period of 12 weeks significantly increased feed intake (12%) in growing lambs, while a 36% increase in average growth rate was recorded. These data indicate that young growing animals are able to respond with an increase in feed intake in a situation where amino acid intake is not sufficient to meet the additional requirements stimulated by GH. This situation is similar to that in lactating cows and ewes where an initial loss of liveweight caused by exogenous GH is stabilized and then reversed with a subsequent increase in voluntary feed intake (see section **1.3.4 Lactation**).

GH treatment commonly increases circulating concentrations of IGF-I in growing animals. The magnitude of the IGF-I response to GH varies considerably among studies, due to differences in experimental protocols including doses of GH and the mode of administration. Nevertheless, changes in growth and carcass composition across studies are related to the changes in circulating IGF-I concentrations. For example, in growing lambs and calves, small changes in plasma IGF-I concentrations (less than 100%) were associated with no effects on growth or carcass composition (Rosemberg *et al.* 1989; McLaughlin *et al.* 1991; Neathery *et al.* 1991), while several fold increases in the IGF-I concentrations were accompanied by 30-36% increase in growth rate and 10 to 20% changes in carcass composition (Pell *et al.* 1990; McLaughlin *et al.* 1993*a*). As a result, circulating concentrations of IGF-I are often highly correlated with measures of growth performance such as growth rate and carcass composition in both sheep (McLaughlin *et al.* 1993*a*) and cattle (Breier *et al.* 1988*b*; Enright *et al.* 1990; Groenwegen *et al.* 1990). For example, Breier *et al.* (1988*b*) reported a highly significant relationship (r= 0.91) between plasma levels of IGF-I and average daily gain in 15 to 17 month old cattle treated with bGH. In contrast, such a relationship is not evident between circulating concentration of GH and growth rate (Beermann *et al.* 1990). Therefore, it appears likely that the growth-promoting effects of GH in farm animals are largely

mediated by changes in circulating concentrations of IGF-I.

It is well recognized that the appearance of hepatic GHRs is important for IGF-I production and postnatal growth in farm animals including sheep and cattle (see section **1.2.2 Growth hormone receptors (GHR)**). In sheep, GHRs appear just before term (Pratt & Anthony, 1995) or soon after birth (Gluckman *et al.* 1983) and numbers rapidly increase a few days after birth to adult values (Gluckman *et al.* 1983). However, in a recent study with neonatal lambs (Sun *et al.* 1992), chronic treatment of rbGH (0.1 or 0.3 mg/kg LW/day) from the day of birth to 11 weeks of age failed to alter growth rate although it did increase wool production. There is some indication that GH treatment at this early stage of life has little effect on plasma levels of IGF-I in the ruminant. For example, the lambs treated with GH by Sun *et al.* (1992) showed little response in plasma IGF-I concentrations (S. N. McCutcheon, personal communication). Similarly, Groenewegen *et al.* (1990) reported that in the 7-day old calf GH treatment over a period of 13 weeks does not affect circulating concentrations of IGF-I. Such a lack of response in plasma IGF-I concentrations and growth to exogenous GH in neonatal animals suggests that, in these animals, the GHR may exist but not be fully functional. Indeed, Badinga *et al.* (1991) studied the ontogeny of hepatic GHR in cattle and reported that bGH binding to liver membranes is very low between days 2 and 30 of postnatal life and then increases gradually up to day 180. Such a slow development in hepatic GHR may be responsible for the delayed growth response to GH observed in neonatal calves, responses not being apparent until 7 weeks after GH treatment commenced (Groenewegen *et al.* 1990). These data suggest that the effectiveness of GH in postnatal growth may be dependent on age and further emphasize the importance of GHR and IGF-I in the regulation of postnatal growth.

1.3.3 Mammary gland development

Milk production is limited by the number of secretory cells in the mammary gland. Thus, manipulation of mammary cell numbers could impact milk yield directly. It is well established that mammary development prior to or during puberty profoundly affects future milk production in ruminants. For example, overfeeding around this period is associated with a reduction in mammary parenchymal growth in heifers (Sejrsen *et al.* 1982; Petitclerc *et al.* 1984; Stelwagen & Grieve, 1990), sheep (Johnsson *et al.* 1986)

and goats (Bowden *et al.* 1995), despite the increased size of mammary glands in overfed animals. This often leads to a substantial reduction in milk production throughout the productive life of cows (Little & Kay, 1979; Harrison *et al.* 1983).

Sejrsen *et al.* (1983) have reported that the decreased mammary parenchymal tissue observed in overfed dairy heifers is associated with decreased serum levels of GH, suggesting that a reduction in secretory tissues during overfeeding may be partly mediated by a change in circulating concentrations of GH. This observation has led several groups to examine whether chronic treatment with GH prior to puberty has any beneficial effects on mammary gland development in heifers (Sejrsen *et al.* 1986; Sandles *et al.* 1987) and sheep (Johnsson *et al.* 1986). Results have shown that GH treatment around this period leads to increases in mammary parenchymal DNA in both species. However, despite this enhanced mammary gland development, no responses in milk yield have been observed during the subsequent lactations of treated animals (Sandles *et al.* 1987; Murphy *et al.* 1991). These data suggest that the enhanced mammary gland development following GH treatment around puberty may not persist into the subsequent lactation period.

Although mammary growth prior to puberty is considered to be critical for subsequent lactation, the majority of mammary gland development occurs during gestation. For example, in sheep, 78% of total mammary growth occurs during this period (Anderson, 1975). This fact led Stelwagen and his co-workers (1992) to investigate a possible mammogenic role of GH during late gestation, a time at which most of the secretory tissue is formed (Swanson & Poffenbarger, 1979). In their study, Holstein heifers were treated with two doses of GH (20 or 40 mg/animal/day) during the last month of gestation and milk yields were measured throughout the subsequent lactation period. Results showed that prepartum treatment with 20 mg of GH significantly increased cumulative milk yields after day 90 of lactation. A similar result has also been reported in ewes where daily injection of GRF from d 105 to 115 of gestation increased milk production during week 5 and 6 of lactation (Kann *et al.* 1988). While these data suggest that GH treatment may stimulate mammogenesis during gestation, Simpson *et al.* (1992) reported that administration of GRF for seven days beginning day -11 from parturition had no effect on subsequent milk yield in primiparous beef heifers. Therefore, further research is required before a definitive

conclusion is reached.

1.3.4 Lactation

It has been well established from a great number of short- and long-term experiments that exogenous GH has a positive effect on milk production in dairy cows (Bauman & McCutcheon, 1986; Peel & Bauman, 1987), sheep (Hart *et al.* 1985; McDowell *et al.* 1987*a*, 1988*a*,*b*; Sandles *et al.* 1988) and goats (Hart *et al.* 1980; Mepham *et al.* 1984; Prosser *et al.* 1991). This "galactopoietic" effect of GH occurs within a few days of GH treatment and returns to control levels shortly after termination of GH administration. The magnitude of the milk yield response to GH varies considerably among studies, ranging from (rarely) 0% up to 40% (Bauman & McCutcheon, 1986; Peel & Bauman, 1987). Much of the variation in milk yield responses to GH may be explained by the nutritional status of treated animals since the highest milk yield response to GH is observed in dairy cows fed a high net energy (NE)/high crude protein (CP) diet relative to those fed lower NE/CP diets (Austin *et al.* 1991; McGuire *et al.* 1992*a*). In addition, the magnitude of response may be influenced by stage of lactation (McDowell *et al.* 1988*a*), genetic merit (Michel *et al.* 1990), environment (Manalu *et al.* 1991) and purity and dose of hormone (Bauman *et al.* 1985).

In most short-term studies, where GH has been administered over several days to a few weeks (Bauman & McCutcheon, 1986; Johnsson & Hart, 1986), the increase in milk yield occurs in the absence of a change in feed intake. As a result, GH treatment results in a large improvement of feed efficiency for milk production (and one which, at least initially, overestimates the true biological effect). However, such an increase in milk production in dairy cows (Bauman *et al.* 1985; Peel *et al.* 1985; Gibson *et al.* 1992) and ewes (Sandles *et al.* 1988) is followed by a gradual increase in feed intake when treatment with GH is extended over a prolonged period. For example, in high producing dairy cows, GH treatment gradually increases feed intakes 5 weeks after commencement of treatment (Bauman *et al.* 1985). These elevated levels of feed intake are sustained for several days after termination of GH treatment, whereas milk yield decreases rapidly over the same period (Sandles *et al.* 1987). These data further emphasize the concept that GH treatment increases the efficiency of feed utilization for milk production simply by diluting maintenance energy requirements. This is further

supported by the earlier observations that neither the digestibility of dietary energy, carbon and nitrogen (Tyrrell *et al.* 1982; Sandles *et al.* 1988), nor the maintenance requirement or efficiency of energy use for milk synthesis (Eisemann *et al.* 1986*b*), is altered by GH treatment.

The observation that GH treatment increases milk yields immediately, but does not increase feed intake until after several weeks of treatment, suggests that in the initial stages of treatment the requirements for extra nutrients must be met by mobilization of body stores. Consistent with this, animals treated with GH have been shown to lose liveweight initially (Bauman *et al.* 1985; Peel *et al.* 1985; Sandles *et al.* 1988). However, as GH treatment progresses, voluntary feed intake then increases to meet additional demands of the mammary gland and the rate of live weight loss is first stabilized and then reversed. As a result, in the majority of long-term studies, liveweights of GH-treated animals have been shown to be similar to those of corresponding control animals at the end of treatment (Bauman *et al.* 1985; Peel *et al.* 1985; Sandles *et al.* 1988) except when very high doses of GH are administered (Soderholm *et al.* 1988; Oldenbroek *et al.* 1989). This replenishment of body stores appears to be crucial in preparation for subsequent lactation since prevention of such an occurrence leads to a substantial decrease in milk response to GH during the subsequent lactation (Chilliard *et al.* 1991).

Metabolic changes observed during GH administration are similar to those occurring in early lactation when nutrients are mobilized from body stores to meet the increased demands of the mammary gland. These orchestrated or coordinated changes in nutrient partitioning, referred to as "homeorhesis" (Bauman & Currie, 1980), have been considered to be crucial in supporting the increased demands of the mammary gland during early lactation. Therefore, GH appears to increase milk yield by affecting nutrient partitioning via homeorhetic signals. The most convincing evidence for GH acting as a homeorhetic signal to alter nutrient partitioning was provided by McDowell *et al.* (1987*a*). In their study, the uptake of key metabolites was measured simultaneously in the hind limb muscle and mammary gland of GH-treated cows. The results showed that GH enhances mammary uptake of glucose and non-esterified fatty acids (NEFA) while reducing uptake of these nutrients by muscle. A similar result has also been reported in lactating ewes in which GH exerts direct effects on the partitioning of key nutrients

between skeletal muscle and mammary tissues (McDowell *et al.* 1988*b*).

One of the most potent metabolic effects of GH is its ability to decrease fat synthesis and stimulate lipolysis in adipose tissue (Vernon, 1989). The specific effects of GH on fat metabolism depend on the energy status of the animals. When animals are in positive energy balance, changes in lipogenesis predominate (Bauman & McCutcheon, 1986). In contrast, when animals are in negative energy balance, the alterations in fat metabolism are primarily related to an increase in lipolysis (Bauman & McCutcheon, 1986). This situation is reflected by chronic elevations in circulating concentrations of NEFA when animals are treated with exogenous GH (Bauman *et al.* 1985; Bauman *et al.* 1988; Sandles *et al.* 1988). As a result, GH treatment leads to a substantial decrease in body fat content but has little effect on other components such as protein and water (Chilliard *et al.* 1991; McGuffey *et al.* 1991).

The mechanism by which GH alters fat metabolism involves alterations in the responsiveness of adipose tissue to homeostatic signals, such as insulin. An *in vitro* study with porcine adipose tissue (Walton & Etherton, 1986) showed that incubation for 50 h with pituitary-derived or recombinant GH decreases the responsiveness of adipose tissue to insulin. Such an anti-insulin effect of GH *in vitro* has been confirmed under *in vivo* conditions, in which GH treatment of growing steers (Dunshea *et al.* 1995) or lactating cows (Sechen *et al.* 1990) decreases responsiveness of adipose tissue to insulin. In addition to its anti-insulin effect, GH treatment appears to increase lipolysis by altering the responsiveness of adipose tissue to hormones such as catecholamines. For example, in lactating cows (McCutcheon & Bauman, 1986; Sechen *et al.* 1990), the maximal response of circulating NEFA and glycerol concentrations to an epinephrine (adrenaline) load was markedly increased during GH treatment. Such an enhanced lipolytic response to epinephrine may be associated with changes in both sensitivity and responsiveness to adenosine since GH treatment reduces the ability of adenosine to inhibit lipolysis in adipose tissue of lactating cows (Lanna *et al.* 1995).

The increased mobilization of lipid stores following administration of exogenous GH results in the sparing of key nutrients, particularly glucose. For example, in lactating cows (McDowell *et al.* 1987*a*; Bauman *et al.* 1988) and ewes (McDowell *et al.* 1988*b*), GH treatment has been shown to reduce oxidation of glucose in both the whole body and the mammary gland while increasing that of NEFA. Such a reduced oxidation of

glucose is also partly mediated via anti-insulin actions of GH since GH treatment reduces the responsiveness and sensitivity of other tissues, including liver and muscle, to insulin in lactating cows (Sechen *et al.* 1990) and growing steers (Dunshea *et al.* 1995). The reduced glucose oxidation, together with a concomitant increase in glycogenesis in the liver (Leenanuruksa & McDowell, 1988), results in the sparing of glucose carbon for lactose synthesis in the mammary gland. This conservation of glucose during lactation is important in view of the high glucose demand of the lactating mammary gland, which can utilize some of 70% of total glucose entry (Bickerstaffe *et al.* 1974; Oddy *et al.* 1985).

The adaptations in whole body metabolism due to GH administration to lactating ruminants might be expected to alter milk composition. However, the concentrations of major nutrients in milk are largely unaltered when animals are in positive nutritional balance (Bauman & McCutcheon, 1986). A small change in fat (increased) or protein (decreased) content occurs when animals are in negative energy/protein balance.

In addition to the alterations in whole body metabolism, GH treatment has been shown to increase mammary blood flow in lactating cows (McDowell *et al.* 1987*a*; Davis *et al.* 1988*b*), sheep (McDowell *et al.* 1988*b*) and goats (Hart *et al.* 1980; Mepham *et al.* 1984). This increased mammary blood flow could further facilitate the supply of key nutrients to the mammary gland. Indeed, in some instances it would appear that the increase in mammary uptake of some precursors is accounted for solely by an increase in mammary blood flow (Davis *et al.* 1988*b*; McDowell *et al.* 1988*b*; Miller *et al.* 1991). However, it remains unclear whether the increase in mammary blood flow is a cause or consequence of the increase in mammary activity, although an increase in metabolic activity in other tissues such as a skeletal muscle can cause localized vasodilation (Milnor, 1980).

Although GH treatment increases blood supply, and thus increases nutrient supply to the mammary gland, such an increasing availability of nutrients to the lactating gland will be useless without a concomitant increase in the mammary gland function. This situation is well demonstrated in an earlier report (Peel *et al.* 1982) in which infusion of additional nutrients postruminally to lactating cows had little effect on milk volume or composition. These data suggest that GH treatment not only increases the delivery of nutrients but also enhances the mammary gland capacity to synthesize milk

components. This suggestion has been substantiated by the increased extraction of glucose and NEFA from the blood by the lactating mammary gland (McDowell *et al.* 1987*a*, 1988*b*; Davis *et al.* 1988*a*; Sandles *et al.* 1988).

It is generally accepted that the mammary gland contains no GHR (Akers, 1985) although low levels of mRNA for the GH receptors were observed in mammary gland of cows (Hauser *et al.* 1990), sheep, pigs, and rabbits (Jammes *et al.* 1991). These data, together with the observation that direct GH infusion in the perfused mammary gland fails to stimulate milk synthesis *in vitro* (Gertler *et al.* 1983) and *in vivo* (McDowell *et al.* 1987*b*), suggest that the effect of GH on the mammary gland is likely to be indirect and mediated by other factors. One of the potential candidates for such a role is IGF-I. It is well recognized that GH treatment increases circulating concentrations of IGF-I in lactating ruminants including cattle (Davis *et al.* 1987; Cohick *et al.* 1989; Sharma *et al.* 1994; Armstrong *et al.* 1995) and goats (Prosser *et al.* 1991). In numerous cases (Cohick *et al.* 1989; McGuire *et al.* 1992*a*; Sharma *et al.* 1994; Armstrong *et al.* 1995), the magnitude of the change in IGF-I concentrations is closely followed by changes in milk yield. Since ruminant mammary glands express abundant mRNA for IGFs receptors (Dehoff *et al.* 1988) and synthesize and secrete IGF-I (Campbell *et al.* 1991*a*), the possibility is raised that IGF-I may stimulate mammary gland activity in the same manner that it stimulates postnatal growth. Direct evidence for such a role of IGF-I in galactopoiesis has been provided by Prosser and his co-workers (1990, 1992) who demonstrated that a direct infusion of IGF-I into the pudendal artery of lactating goats led to a 15-25% increase in milk secretion relative to that in untreated glands. In contrast, Davis *et al.* (1989*a*) reported that IGF-I had no effect on milk production when infused into the jugular vein of lactating goats. However, the effects of dilution into the general circulation and possible sequestration of IGF-I by IGFBPs in the serum may be responsible for the lack of effect observed in this latter study. Therefore, the role of IGF-I in GH-stimulated galactopoiesis is unclear.

1.4 PLACENTAL LACTOGEN (PL)

Placental lactogen (PL) is a member of the GH/PRL family based on structural and functional similarity (Warren *et al.* 1990*a,b*). The occurrence of this hormone has been demonstrated in humans, rats, cattle, sheep and goats although some species, such as

pigs, cats, dogs, rabbits and horses, do not produce this peptide (Byatt *et al.* 1992*c*). Because of its structural similarity to GH and PRL, PL has been considered to possess somatogenic and lactogenic activities.

1.4.1 Nature and secretion of PL

Placental lactogen is a peptide which is secreted by the placenta. It is recognized that human PL (hPL) is synthesized and secreted by the syncytiotrophoblastic tissue (McWilliams & Boime, 1980; Hoshina *et al.* 1982). In contrast, there has been some disagreement with regard to the exact location of oPL production. While some studies have identified chorionic primary cells as the site of oPL production (Martal *et al.* 1977; Carnegie *et al.* 1982), others determined it to be a product of chorionic binucleate cells (Watkins & Reddy, 1980; Wooding, 1981). This problem has been resolved by a recent study (Kappes *et al.* 1992) in which the concentrations and cellular location of oPL mRNA expression of the chorionic cell type(s) during mid- and late-gestation were measured by immunocytochemistry and *in situ* hybridization. Results have shown that the chorionic binucleate cell is the sole source of oPL.

In sheep, oPL is first detectable in maternal plasma by day 40 to 60 of gestation (Handwerger *et al.* 1977; Gluckman *et al.* 1979*b*; Butler *et al.* 1981; Kappes *et al.* 1992), reaches a maximum value at day 120 to 140 day of gestation, and declines thereafter (Handwerger *et al.* 1977; Gluckman *et al.* 1979*b*; Kappes *et al.* 1992). Changes in plasma concentrations of oPL correspond with changes in oPL gene transcription throughout gestation (Kappes *et al.* 1992). *In situ* hybridization studies (Kappes *et al.* 1992) have shown that the concentration of oPL mRNA in individual binucleate cells remains constant until the last week of gestation, suggesting that the increase in the total amount of oPL mRNA seen as gestation progresses is due to an increase in the number of binucleate cells in the placenta. This is in agreement with data showing that the proportion of binucleate cells increases in the chorionic epithelium during gestation (Kappes *et al.* 1992). This increase in binucleate cell number is at the expense of chorionic connective tissue. A similar situation is also apparent in humans in which the syncytiotrophoblastic tissue, the site of the hPL production, also increases in size as gestation progresses and is thought to be responsible for the 5-fold increase in hPL mRNA concentrations between the first and third trimesters (Boime *et al.* 1982).

While concentrations of oPL in maternal plasma increase until late gestation, concentrations of oPL in fetal plasma reach a peak at mid-gestation (Handwerger *et al.* 1977; Gluckman *et al.* 1979*a*; Kappes *et al.* 1992). Such a different peak interval in oPL levels between maternal and fetal plasma suggests that release of oPL is controlled differently on the fetal and maternal sides of the placenta. This is further manifested by different patterns of plasma concentrations of oPL between fetal and maternal sheep. For example, the concentrations of oPL in fetal plasma are relatively constant across time and between individual animals, whereas the concentrations of oPL in maternal plasma vary considerably over the same parameters (Schoknecht *et al.* 1991; Bauer *et al.* 1995). It has been suggested that secretion of oPL into the fetal circulation may result from a population of binucleate cells that do not migrate into the maternal-fetal interface of the placentome (Kappes *et al.* 1992). This is corroborated by the observation that the concentration of oPL mRNA in fetal cotyledonary tissue, like plasma fetal oPL levels, increases from day 60 to 120 of gestation and then plateaus.

1.4.1.1 Chemical structure of PL

Placental lactogen is a single polypeptide hormone of about 22,000 daltons. It is composed of 191 amino acids with two intrachain disulfide bridges. The amino acid sequence of placental lactogen was first determined in humans (Shine *et al.* 1977) and subsequently in other species including rodents (Duckworth *et al.* 1986; Jackson *et al.* 1986), sheep (Colosi *et al.* 1989; Warren *et al.* 1990) and cattle (Schuler *et al.* 1988). The primary structure of PL is similar to that of related pituitary hormones such as GH and PRL. In most species, including sheep and cattle, the primary sequence of PL is closer to that of PRL than to that of GH. For example, in sheep, mature PL has significant amino acid sequence identity with PRL (48-49%) (Colosi *et al.* 1989; Warren *et al.* 1990*b*) and structure similarity with PRL with regard to the location and number of disulphide bridges within the molecule (Caridad & Wolfenstein-Todel, 1988). These data suggest that PLs and PRLs in most species have evolved from a common gene. The exception is human PL which has only limited homology with prolactin, but very high homology with hGH mRNA coding sequence (Shine *et al.* 1977). Thus, it is likely that the gene for hPL, unlike those for other PLs, has evolved from the GH gene family rather than the PRL gene family.

The amino acid composition of PL also shows considerable differences among species. For example, amino acid sequence identity between oPL and bPL is less than 67% (Warren *et al.* 1990*b*). This interspecies similarity is not as great as that which exists between oPRL and bPRL (98% identity; Sasavage *et al.* 1982) or between oGH and bGH (more than 99% identity; Chene *et al.* 1989). The fact that PL in sheep and cattle is not as highly conserved as its related hormones (PRL and GH) suggests that this molecule may have divergent biological roles in these two species. This possibility is further raised by the observation that oPL is non-glycosylated (Warren *et al.* 1990*a,b*), whereas bPL is heavily glycosylated (Byatt *et al.* 1990). Such an interspecies divergence in primary amino acid sequence for PLs between sheep and cattle is also apparent in other species, suggesting the same rule may be applied in other species.

Placental lactogen also has some degree of micro-heterogeneity within a species. The existence of multiple forms of PL was reported first in human (Chard, 1983) and subsequently in other species including sheep (Chan *et al.* 1986) and cattle (Byatt *et al.* 1986, 1987). These variants may be due to posttranslational modification, such as glycosylation, although enzymatic deglycosylation of bPL has little effect on receptor binding or biological activity in both somatotropin radioreceptor assay (mouse preadiposite) and lactogenic bioassay (Nb_2 lymphoma cell) (Byatt *et al.* 1990). However, there is also some evidence to suggest that the expression of multiple products may be due to alternate splicing and allelic variants of the PL gene (Warren *et al.* 1990*b*; Krivi *et al.* 1989; Kessler *et al.* 1991). For example, two distinct cDNAs for bPL that differ by 350 base pairs in the length of their 5' untranslated region have been isolated (Krivi *et al.* 1989).

Although PLs are more closely related to PRL rather than to GH in most species (except human), this similarity is based solely on structure and is not a reflection of functional identity. For example, oPL competes effectively with GH for its binding site on fetal and maternal sheep liver (Breier *et al.* 1994*a,b*), and to human GH receptors (Colosi *et al.* 1989), despite being structurally related to PRL (Warren *et al.* 1990*b*). It is clear that in order to obtain relevant information, biological studies must be carried out in homologous systems. This is evidenced by studies in which oPL was shown to be a potent stimulator of secretory activity in rabbit mammary explants, but had little effect on ovine mammary tissue (Severley *et al.* 1983).

1.4.1.2 Regulation of PL synthesis and secretion

The specific hormonal and metabolic factors that regulate the secretion of PLs, including oPL, are poorly understood. In sheep, acute decreases or increases in plasma NEFA concentrations have little effect on circulating concentrations of oPL (Butler *et al.* 1987). Although acute changes in plasma glucose concentrations have been shown to modulate hPL secretion (Burt *et al.* 1970; Prieto *et al.* 1976), insulin-induced acute hypoglycaemia, or hyperglycaemia, failed to alter plasma oPL levels in sheep (Butler *et al.* 1987). A similar result has been also reported in chronic conditions in which fasting of pregnant ewes for 72 h had little effect on maternal oPL levels (Bulter *et al.* 1987). In contrast, in another study with pregnant ewes, a similar period of prolonged fasting increased circulating concentrations of oPL in both fetal and maternal sheep (Brinsmead *et al.* 1981). This situation is further demonstrated in a recent study with pregnant ewes (Bauer *et al.* 1995), in which severe undernutrition (25% of the recommended energy and protein requirement) over a prolonged period tended to increase circulating concentrations of oPL in restricted dams but decrease concentrations of oPL in fetal sheep. Therefore, the effect of nutritional alteration on oPL secretion remains unclear. A similar situation also exists with regard to the effect of bromocriptine on the secretion of PLs. For example, some studies have reported that administration of bromocriptine (CB154) reduces the maternal concentrations of PL in sheep (Lowe *et al.* 1979) and goats (Buttle *et al.* 1979), while others found no such effect (Martal & Lacroix, 1978; Forsyth *et al.* 1985).

Some of the inconsistent results observed in studies with metabolic manipulation and CB154 treatment may be partly related to the fact that maternal levels of PL in sheep and goats fluctuate widely in successive hourly samples (Hayden *et al.* 1980; Taylor *et al.* 1980). For example, in hourly sampling from pregnant goats (Hayden *et al.* 1983) or sheep (Butler *et al.* 1980), plasma concentrations of PL fluctuated by between 30 and 100%. A similar situation also occurs in humans, in which PL concentrations fluctuate by 15-100% in successive blood samples (Vigneri *et al.* 1975; Zlatnik *et al.* 1979). Such large variations in PL concentrations over a short period of blood sampling suggest that intensive and prolonged blood sampling regimens are required to determine accurately the actual profile of PL. This situation is further complicated by a recent report that preexisting maternal nutritional status affects the response of plasma PL concentrations

to acute starvation in both fetal and maternal sheep (Oliver *et al.* 1992).

1.4.2 Receptors

It has been generally assumed that PLs exert their actions through binding to either GH or PRL receptors. However, there is some evidence to suggest that PLs actually exert their biological actions via their own receptors. Such a possibility was first raised by Chan *et al.* (1978) who reported that specific binding sites for PL were found in number of fetal as well as postnatal tissues. Since then, the presence of such distinct receptors for PLs has been identified in several species including sheep (Freemark *et al.* 1986, 1988), cattle (Galosy *et al.* 1991; Kessler *et al.* 1991) and humans (Hill *et al.* 1988).

Specific binding sites for PL have been normally identified by binding studies using either GH or PRL as a competitive ligand. These competitive binding studies have shown that binding sites for PL bind PL more potently than GH or PRL. For example, Freemark *et al.* (1986) reported that the potency of oPL (K_d=0.27 nM) in competing for binding sites in fetal ovine liver was 90 and 1,300 times greater than that of oGH and oPRL, respectively. In sheep, these binding sites for PL are first detected as early as day 70 of gestation and increase in number several fold throughout gestation, reaching a maximum value at 3-7 days before parturition (Freemark *et al.* 1986). Similarly, in humans, specific receptors for PL in the liver and skeletal muscle are detected as early as 48 days of gestation although, in contrast to fetal lambs, there are also receptors for GH in the liver at this stage (Hill *et al.* 1988). In both species, the appearance of these specific binding sites is consistent with specific biological actions of PL in fetal tissues, suggesting that the distinct biological actions of PL are mediated via its own specific receptors. This suggestion is corroborated by the observation that oGH or oPRL do not bind to fetal ovine hepatic membranes (Freemark *et al.* 1986). Evidence for a unique PL receptor is further provided by the observation that treatment of microsomal fractions of fetal and maternal sheep liver with Triton X-100 solubilizes the oPL receptor but not the oGH receptor (Freemark *et al.* 1988). It therefore appears that the PL receptor, together with the GH and PRL receptors, constitutes a family of distinct but related hormone receptors that differ in their relative affinities for PL, GH and PRL. Changes in the expression of the PL, GH, and PRL receptors may mediate changes in the

hormonal control of growth during the transition from fetal to postnatal life (Freemark *et al.* 1986).

Although the existence of specific receptors for PL has been identified in fetal and maternal tissues, there is also increasing evidence to suggest that biological actions of PL observed in postnatal animals may be mediated via the same receptor as GH. Such a possibility was first raised by ligand binding studies which showed that hepatic oGHR binds both oGH and oPL with high affinity in Chinese hamster ovary or COS cells transfected with complementary DNA clones of the full-length transmembrane oGHR (Breier *et al.* 1992; Fiddes *et al.* 1992). Consistent with this, more recent ligand binding studies of oPL and oGH to hepatic microsomal membranes in the lamb show shows parallel ontogeny (Breier *et al.* 1994*a*). These observations are further supported by recent studies, in which the sheep oPL-receptor complex was shown to be strongly recognized by antibodies raised against the extracelluar region of the rabbit GHR (Breier *et al.* 1994*b*). These data strongly indicate that PL and GH interact with a common receptor. This is further substantiated by recent *in vivo* experiments in which administration of recombinant bPL stimulated the growth of rats in a manner similar to bGH (Byatt *et al.* 1991). A similar result has been also reported with recombinant oPL in GH-deficient dwarf rats (Singh *et al.* 1992).

The mechanism which regulates expression of the PL receptor remains largely unexplained. However, there is increasing evidence that nutrition plays a major role. For example, fasting of pregnant ewes for 72 h in late gestation causes a 50-75% reduction in the specific binding of oPL to maternal and fetal liver (Freemark *et al.* 1989, 1990). These effects of fasting are reversed entirely by refeeding. The decrease in oPL binding during fasting is associated with a large reduction in the number of binding sites. There are no changes in the affinity of the hepatic receptor for oPL, the subunit structure of the receptor, or the extent of occupancy of the receptor *in vivo* by endogenous maternal and fetal hormones. The numbers of PL binding sites in maternal and fetal liver correlate positively with maternal and fetal plasma concentrations of glucose and insulin, suggesting that both factors may regulate PL binding in sheep liver. This suggestion is partly supported by a recent study (Freemark *et al.* 1992) in which a down-regulation of oPL receptors in the fetal liver during fasting was reversed completely by a direct infusion of glucose into the fetus. However, glucose and insulin have no effect on PL

binding to ovine fetal fibroblasts *in vitro*, suggesting that their effects on hepatic PL receptors in the fetal sheep are tissue specific and/or mediated indirectly through effects on other hormones, nutrients or growth factors (Freemark *et al.* 1992). Fasting of pregnant ewes also stimulates a 2-fold increase in fetal plasma oPL concentrations, and fetal plasma oPL levels are inversely related to the number of PL receptors in fetal liver (Freemark *et al.* 1992). These observations suggest that oPL may down-regulate the number of PL-binding sites in fetal tissues. Whatever the mechanisms, it appears that glucose and other nutritional factors regulate the expression of the PL receptor in fetal and maternal sheep liver. Alterations in PL binding may play a significant role in the metabolic adaptation of the mother and fetus to nutritional deprivations and stress. For example, the reduction in fetal hepatic PL receptors during maternal fasting may contribute to the mobilization and depletion of fetal liver glycogen stores and may play a role in the pathogenesis of the fetal growth retardation that accompanies maternal energy deprivation.

1.4.3 Biological actions of PLs

Since PLs have considerable homology with GH and PRL, the possibility is raised that they may have potential in the manipulation of farm animal production. Although ruminant PLs have been shown to have both somatogenic and lactogenic properties in heterologous systems, such structural and functional relationships have not been fully examined in homologous systems.

1.4.3.1 Fetal development and maternal metabolism

There is some evidence to indicate that PLs, including oPL, may be involved in the regulation of fetal growth. For example, oPL promotes amino acid transport in fetal rat muscle (Freemark & Handwerger, 1983) and stimulates glycogen synthesis while inhibiting glucagon-induced glycogenolysis in fetal rat hepatocytes (Freemark & Handwerger, 1984, 1985). Although these activities observed in the rat do not necessarily translate to the homologous species, a more recent study has demonstrated that oPL also stimulates glycogenesis in ovine fetal hepatocytes (Freemark & Handwerger, 1986). Similarly, hPL has been shown to stimulate amino acid transport, DNA synthesis, mitogenesis (Hill *et al.* 1986) and [^{3}H] thymidine incorporation (Hill *et*

al. 1985) in isolated human fetal fibroblasts and myoblasts. Consistent with these data, there is a close relationship between fetal circulating concentrations of PL and fetal weight in sheep (Schoknecht *et al.* 1991) and humans (Hill *et al.* 1988).

Adams *et al.* (1983) reported that oPL stimulates IGF-I production in rat fibroblasts. Such a stimulatory effect on IGF-I also occurs when isolated human fetal myoblasts and fibroblasts are exposed to hPL (Hill *et al.* 1985). These data suggest that the somatogenic effect of PL observed in fetal tissue may be associated with enhanced IGF-I production. This suggestion is further supported by the observation that the mitogenic effect of hPL on fetal fibroblasts is inhibited by an antibody directed against IGF-I (Hill *et al.* 1986). The mechanism by which PL stimulates IGF-I production remains unclear. However, it is unlikely that IGF-I secretion in fetal tissues is mediated by GHR since fetal GHRs exist but are not functional (see section **1.3.1 Fetal development and maternal metabolism**). This is further strengthened by the observation that GH has no or little biological effects on fetal tissues although it has somatotrophic and metabolic effects on postnatal tissues. Rather, the specific effects of PL on fetal tissues suggest that PL may have biological actions via its own specific receptors. Consistent with this, Freemark *et al.* (1992) reported that the number of PL binding sites (oPL receptors) in fetal sheep liver was positively correlated with fetal weight. Similarly, in humans, the weight of the fetus in early and mid-gestation was positively correlated with the hPL-binding capacity of the fetal liver (Hill *et al.* 1988). Therefore, the presence of specific, high affinity PL receptors in fetal tissue provides a mechanism whereby PL may function as a growth hormone in the fetus(es).

Several studies have also reported that there is a close relationship between maternal circulating concentrations of oPL and litter size (Gluckman *et al.* 1979*b*; Hayden *et al.* 1979; Oddy & Jenkin, 1981), suggesting that oPL may be involved in the regulation of fetal growth through effects on maternal metabolism. Metabolic studies using a placental extract containing oPL have suggested that oPL reduces maternal glucose utilization and increases the catabolism of triglycerides while promoting maternal utilization of free fatty acids as a primary fuel (Thordarson *et al.* 1987). This glucose-sparing effect may increase glucose supply to the conceptus and thus promote fetal growth. While some studies have supported this concept (Oddy & Jenkin, 1981; Regnault *et al.* 1993), other studies have not (Handwerger *et al.* 1976; Waters *et al.* 1985). The conflicting results

in some of these studies may be partly associated with differences in experimental protocols such as length of treatment and stage of gestation. For example, Thordarson *et al.* (1987) infused a placental extract enriched in oPL for 36 h, whereas Handwerger *et al.* (1976) infused a partially purified oPL only for 8 h. Similarly, infusion of an antibody against circulating oPL was continued only for 12 h in another study (Waters *et al.* 1985). It has been reported from *in vitro* studies that a minimum period of 4-8 days is required for lactogenic hormones, including PLs, to have their maximum effect on insulin and glucose secretion from pancreatic cells (Sorenson *et al.* 1987; Brelje *et al.* 1993). These *in vitro* results may indicate that metabolic actions of lactogenic hormones are chronic rather than acute. Furthermore, in one of the studies (Waters *et al.* 1985), infusion of an antibody against plasma oPL was performed at day 131 of gestation. At this stage the fetal pituitary appears to be functional and thus fetal growth may be less dependent on maternal circulating concentrations of oPL. Recently, Parsons *et al.* (1992) used the incorporation of 5-Bromo-2'-deoxyuridine (BrdU) to assess islet cell proliferation and pancreas perfusion to evaluate insulin secretion throughout pregnancy in the rat. Results showed that rPL enhanced islet cell proliferation and insulin secretion until day 14 of gestation, but such effects disappeared thereafter. The lack of effect of rPL on islet function and insulin secretion in late gestation suggests that biological actions of PL may be greatly diminished during the later stages of gestation. Therefore, lack of evidence for a positive role of oPL in maternal metabolism in some of the studies may be due simply to the short length of infusion as well as the timing of infusion.

1.4.3.2 Postnatal growth

Several studies have shown that PLs have biological actions in postnatal tissues that are similar to those of GH. For example, oPL, which binds to GH receptors in the sheep liver with an affinity comparable to that of oGH (Chan *et al.* 1978; Emane *et al.* 1986; Freemark *et al.* 1986), is equipotent to bGH in stimulating ornithine decarboxylase activity (Butler *et al.* 1978) and amino acid transport (Freemark & Handwerger, 1982) in postnatal tissue of rats. Consistent with *in vitro* results, administration of partially purified or recombinant oPL stimulates IGF-I production (Hurley *et al.* 1977; Singh *et al.* 1992) and weight gain in hypophysectomized (Hurley *et al.* 1977) and growth

hormone-deficient dwarf (Singh *et al.* 1992) rats with a similar or superior potency to bGH. Such a growth-promoting effect of PL observed in GH-deficient animals is further demonstrated in normal rats with an intact somatotrophic axis, in which recombinant bPL stimulated growth rate with a potency equal or superior to that of bGH (Byatt *et al.* 1991).

Although the above studies clearly demonstrate that PL has somatogenic activities, it remains unclear whether the growth-stimulating actions of ruminant PLs observed in the rat can be translated into the homologous species. Recently, McLaughlin *et al.* (1993*a*) studied biological actions of recombinant bPL (4 and 8 mg/d) in finishing lambs over a prolonged period and compared results to those of bGH at comparable doses (4 and 8 mg/d). Results showed that growth, carcass composition, and plasma concentrations of IGFs and insulin were not affected by bPL. In contrast, bGH significantly increased growth performance as well as plasma IGF-I levels. These data suggest that great caution must be exercised when interpreting results observed in the heterologous system.

1.4.3.3 Mammary gland development

The development of the mammary gland is primarily regulated by the ovarian steroids (Cowie *et al.* 1980; Purup *et al.* 1993), but the presence of a lactogenic hormone such as prolactin is required for full lobular-alveolar growth to occur (Cowie *et al.* 1980). Thus, administration of oestrogen and progesterone to non-pregnant ewes (Schams *et al.* 1984) or heifers (Schams *et al.* 1984) will stimulate mammary growth, but mammogenesis is severely restricted if prolactin secretion is inhibited by simultaneous treatment with CB154. However, in pregnant goats (Buttle *et al.* 1979; Forsyth *et al.* 1985) and sheep (Delouis *et al.* 1980; Schams *et al.* 1984) mammary gland development was largely unaffected by hypophysectomy or CB154 treatment which resulted in a suppression of prolactin release. These data suggest that, in goats and ewes, PL is able to maintain mammogenesis in the absence of prolactin.

Further indirect evidence of a role for PL in the regulation of mammogenesis is provided by Currie *et al.* (1977) who reported that the mid-gestation rise in maternal circulating concentrations of PL was paralleled by rapid lobulo-alveolar mammary development in goats. Studies with sheep (Butler *et al.* 1981) and goats (Hayden *et al.* 1979) have also

shown that twin-bearing ewes/does have both higher concentrations of PL in maternal plasma and greater mammary gland development than single-bearing ewes/does. As a result, subsequent milk yields in goats are positively correlated with PL concentrations in maternal plasma during late pregnancy (Hayden *et al.* 1979) and the same relationship may be inferred for ewes (Butler *et al.* 1981).

While there is considerable circumstantial evidence to indicate that PL has a stimulatory effect on mammary growth in sheep and goats, such a role of PL remains unclear in cattle. This situation may be associated with the very low levels of PL in cattle (Byatt *et al.* 1987; Wallace, 1993). For example, Wallace (1993) reported that the highest concentration of bPL measured in maternal serum was 2.93 ng/ml which is much lower than levels of PL (2 ug/ml) observed in sheep (Handwerger *et al.* 1977) and goats (Currie *et al.* 1990). However, mammogenesis in pregnant heifers occurs in the presence of CB154 (Schams *et al.* 1984), as noted in sheep and goats. Furthermore, bPL has been shown to stimulate DNA synthesis of bovine mammary tissue maintained in athymic nude mice (Vega *et al.* 1989). This result is further supported by a recent *in vivo* study (Byatt *et al.* 1994) in which administration of recombinant bPL for seven days stimulated mammogenesis and lactogenesis in steroid-primed dairy heifers. These data suggest that bPL may have a positive role in the development of the mammary gland in cattle despite its low concentrations in circulation.

The mechanisms by which PL stimulates mammogenesis remain unclear. Although the presence of oPL receptors has been demonstrated in the sheep mammary gland (Chan *et al.* 1978), the concentration of these receptors is very low. In contrast, such receptors are abundant in the liver of pregnant or non-pregnant ewes (Freemark *et al.* 1986; Freemark *et al.* 1988), suggesting that the liver is likely to be the main target for oPL. It is well recognized that PLs, including oPL, stimulate circulating concentrations of IGF-I in postnatal animals (Hurley *et al.* 1977). Since the ruminant mammary gland contains abundant mRNA for IGF-I receptors (Dehoff *et al.* 1988), the enhanced mammogenesis observed in PL-treated animals may be partly mediated by increased production of IGF-I from hepatic and non-hepatic tissues.

1.4.3.4 Lactation

Another property of PL is its lactogenic activity. Servely *et al.* (1983) reported that

oPL potently stimulated lactogenic activity in mammary explants from rabbits in mid-pregnancy. Similarly, bPL has been shown to be a potent stimulator of secretory activity in rabbit mammary explants (Byatt & Bremel, 1986). Although in both studies the lactogenic activity of PL was much less in the homologous system, thus questioning the lactogenic role of PL in ruminants, such galactopoietic activity of bPL has been demonstrated in a recent *in vivo* study with dairy cows (Byatt *et al.* 1992*a*). In that study, Byatt and his co-workers administered several doses of bPL (5, 10, 20, and 40 mg/day) for 9 days to dairy cows in mid-lactation and compared the results to those in bGH-treated (20 mg/day) and saline-treated groups. Results showed that the three highest doses of bPL increased milk yield in a dose-dependent manner, although bPL was much less potent than bGH. This elevated milk yield in bPL-treated animals was accompanied by increased circulating levels of IGF-I, although the magnitude of the IGF-I response to bPL treatment was again small compared to that of bGH treatment. These data suggest that bPL may stimulate milk yield via the same mechanism as bGH does. However, bPL treatment also stimulated voluntary feed intake, an effect which was absent with bGH treatment. Since the increase in energy intake observed in bPL-treated animals was highly correlated with the increased energy secreted as milk (r=0.94), the galactopoietic effect of bPL may be also associated with its stimulation of energy intake.

1.5 PURPOSE AND SCOPE OF THE INVESTIGATION

We have known for many years that GH administration may be used to improve farm animal performance but this has become a commercial reality only in the last decade with the advent of recombinant techniques for production of GH. Since then, our understanding of the biological actions of GH and the mechanisms by which GH acts has been greatly expanded.

While the biological actions of GH are well defined, much less is known about placental lactogen (PL). Although available data have shown that PLs, including oPL, exhibit somatogenic and lactogenic activities, such findings have been observed mainly with partially purified PL or placental extracts. Furthermore, most of the biological actions of PL have been studied in heterologous systems. Therefore, the biological actions of PLs and the mechanisms which by PLs act in farm animals remain largely unknown.

It is also well recognized that IGF-I functions as a key hormone in the somatotrophic axis, mediating many of the actions of GH in an endocrine and paracrine/autocrine manner. Recent studies have suggested that IGF-I may mediate biological actions of oPL, as it does for GH. Nevertheless, IGF-I has not been studied extensively in terms of its potential to enhance productivity of animals with an intact somatotrophic axis. Thus, while recombinant oPL and IGF-I have potential as stimulators of productivity in farm animals, they have not been studied extensively. The objectives of this study were therefore to:

1. Examine the effects of recombinant oPL on mammogenesis, galactopoiesis and growth in sheep and explore the mechanisms by which observed effects might occur;

2. Examine the effects of recombinant IGF-I on the growth and metabolism of sheep whose endogenous IGF-I levels were expected to be low as a consequence of energy restriction, and;

3. Consider the situations in which recombinant oPL and IGF-I might have commercial potential as stimulators of productivity in farm animals.

CHAPTER 2

DIFFERENTIAL EFFECTS OF RECOMBINANT OVINE PLACENTAL LACTOGEN AND BOVINE GROWTH HORMONE ON METABOLISM AND UTERINE DEVELOPMENT IN PREGNANT EWES

bGH treatment at day 101 of gestation stimulated uterine development, whereas such effects were not apparent in oPL-treated ewes.

2.1 ABSTRACT

The effects of recombinant ovine placental lactogen (oPL) and bovine growth hormone (bGH) on maternal metabolism, mammary gland development and fetal growth were examined in singleton-bearing ewes at day 101 of gestation. Ewes were treated by twice daily subcutaneous injection for 7 days with oPL (n=7) or bGH (n=8) at a dose of 0.15 mg/kg liveweight (LW)/day or with saline (n=8). Jugular blood samples were taken on days -1 and 7 of treatment. Relative to those of saline-treated ewes, bGH treatment significantly ($P<0.01$) reduced maternal plasma concentrations of oPL at day 7 of treatment. Administration of bGH also increased maternal circulating concentrations of insulin-like growth factor (IGF)-I ($P<0.001$), insulin ($P<0.01$), glucose ($P<0.01$) and non-esterified fatty acids (NEFA) ($P<0.10$) and decreased IGF-II ($P<0.001$) and urea ($P<0.05$) concentrations relative to those in oPL- or saline-treated ewes. None of these parameters was affected by oPL treatment (vs saline treatment) except for maternal circulating oPL concentration which was increased by 50% ($P<0.001$). Neither bGH nor oPL had significant effects on dimension or trimmed weight of the mammary gland. Similarly, oPL treatment had no effects on weights of uterine components, whereas treatment with bGH increased the total weight of the gravid uterus ($P<0.05$) and weights of the uterine fluids, fetal membranes and myoendometrium ($P<0.05$). oPL, but not bGH treatment, also reduced maternal spleen ($P<0.05$) and heart ($P<0.10$) weights relative to those in saline-treated ewes. These results suggest that oPL acts in a distinctly different manner from bGH and that effects of oPL in the ewe are not mediated via changes in circulating concentrations of either IGF-I or IGF-II.

2.2 INTRODUCTION

Ovine placental lactogen (oPL) is a member of the growth hormone (GH)/prolactin (PRL) family. It is produced by the chorionic binucleate cells of the sheep placenta (Kappes *et al.* 1992) and released into both the fetal and maternal circulations. oPL is first detectable in trophoblastic tissues at day 16 of pregnancy and in maternal plasma by day 40 to 60 of gestation (Gluckman *et al.* 1979*a*; Butler *et al.* 1981; Kappes *et al.* 1992). Concentrations of oPL in fetal plasma peak at day 80 to 120 of gestation and then remain constant or decline until parturition (Gluckman *et al.* 1979*b*; Taylor *et al.* 1980; Kappes *et al.* 1992), whereas concentrations of oPL in maternal plasma peak at

day 120 to 140 of gestation and then decline until parturition (Gluckman *et al.* 1979 *a,b*; Kappes *et al.* 1992).

Recently, oPL has been cloned and sequenced (Colosi *et al.* 1989) and found to share considerable homology with bovine PL (67%), ovine PRL (48 %) and a lesser degree of homology with oGH (28 %) or other species of GH (25 %). The mid-gestation rise in maternal circulating concentrations of PL is paralleled by rapid lobulo-alveolar mammary development in goats (Currie *et al.* 1977). Studies with sheep (Butler *et al.* 1981) and goats (Hayden *et al.* 1979) have also shown that twin-bearing ewes/does have both higher concentrations of PL in maternal plasma and greater mammary gland development than single-bearing ewes/does. As a result, subsequent milk yields in goats are positively correlated with PL concentrations in maternal plasma during late pregnancy (Hayden *et al.* 1979) and the same relationship may be inferred for ewes (Butler *et al.* 1981). Therefore, there is considerable circumstantial evidence to indicate that PL either stimulates or supports mammary growth in sheep and goats. This hypothesis is further supported by a recent study (Byatt *et al.* 1994) in which administration of recombinant bPL for seven days stimulated mammogenesis and lactogenesis in steroid-primed dairy heifers.

The close relationship between maternal circulating concentrations of oPL and litter size (Gluckman *et al.* 1979*a*; Hayden *et al.* 1979) also suggests that oPL may be involved in the regulation of fetal growth through effects on maternal metabolism. Metabolic studies using a placental extract containing oPL have suggested that oPL reduces maternal glucose utilization and increases the catabolism of triglyceride while promoting maternal utilization of free fatty acids as a primary fuel (Thordarson *et al.* 1987). This glucose-sparing effect may increase glucose supply to the conceptus and thus promote fetal growth. Consistent with these results, fetal weight is positively correlated with circulating concentrations of oPL in maternal plasma (Taylor *et al.* 1980; Kappes *et al.* 1992) and oPL concentrations are regulated by maternal nutrition (Gluckman & Barry, 1988).

Although the above studies suggest a stimulatory role of maternal oPL in both mammogenesis and fetal growth, such hypotheses have not been tested directly. This chapter reports the first study to examine the effects of recombinant oPL administered *in vivo* to the homologous species. The study examined effects of oPL administered to

the pregnant ewe on maternal metabolism, mammary gland development and the growth of the conceptus.

2.3 MATERIALS AND METHODS

2.3.1 Animals and treatment

Twenty-four Border Leicester x Romney ewes aged 2 to 5 years were used in this study. All were mated at the second oestrus after progesterone synchronization and single pregnancy was confirmed using ultrasound at days 55 and 75 of pregnancy.

On day 86 of pregnancy, ewes were individually housed on slatted floors. Over the next 15 days they were adapted to a diet which consisted of *ad libitum* sheep nuts and chaffed lucerne hay. Feed was offered at 1500h daily. Intakes of the sheep nuts and lucerne chaff were recorded daily and fresh water was available *ad libitum.*

At the end of the adjustment period the ewes were divided into three groups of 8 each, balanced for liveweight (LW) and age. One group was injected subcutaneously with recombinantly-derived oPL (0.15 mg/kg LW/day; Lot # M3RD78, provided by Dr R Vandlen, Genentech, South San Francisco), one group with recombinantly-derived bGH (0.15 mg/kg LW/day; Lot # 7368C-69Q, provided by Dr IC Hart, American Cyanamid, Princeton, NJ), and the control group with sterile physiological saline (0.036 ml/kg LW/day). This batch of oPL has been shown previously to be potently somatotropic in the GH-deficient rat (Singh *et al.* 1992). The bGH was solubilized at a concentration of 2 mg/ml in carbonate buffered saline (pH 9.4) while the oPL was provided as a 2 mg/ml solution in phosphate buffered saline (pH 7.6). Subcutaneous injections were alternated between the left and right sides of the neck and were administered twice daily, at 0800 and 1600h (half the daily dose at each time), except for the final injection which was administered at 2400h on the day before slaughter. Treatment continued for 7 days (ie days 101 to 107 of pregnancy inclusive). This experiment was approved by the Massey University Animal Ethics Committee.

2.3.2 Blood sampling

Blood samples were taken by jugular venipuncture at 0800, 1100 and 1500 h on days -1 and 7 of treatment (days 100 and 107 of pregnancy). Samples (8 ml) were

withdrawn into vacutainers (Nipro Medical Industries, Tokyo,Japan) containing EDTA as the anticoagulant and immediately placed on ice. Within 30 min the samples were centrifuged at 3000 g and 4°C for 20 min. Plasma was pipetted into duplicate vials and stored at -20°C until assayed.

2.3.3 Chemical analyses

Urea, creatinine, glucose and non-esterified fatty acid (NEFA) concentrations in plasma were determined as described previously (Cottam *et al.* 1992) with intra- and inter- assay coefficients of variation of 1.3 and 3.5, 0.4 and 1.9, 1.2 and 4.9, 2.6 and 3.8%, respectively.

Plasma IGF-I concentration was measured by radioimmunoassay (Breier *et al.* 1994*a*) with intra- and inter- assay coefficients of variation of 4.5 and 7.2%, respectively. IGF-I concentrations are expressed in terms of the international reference recombinant human IGF-I preparation 87/518 (National Institute for Biological Standards and Control, Potters Bar, Herts, U.K.).

Plasma IGF-II concentrations were measured by radioimmunoassay following acid gel chromatography to separate IGF binding proteins (Koea *et al.* 1992). Highly purified ovine IGF-II (batch 6. 11. 87, Dr L. Moore, Wallaceville Agricultural Research Station, Wellington, New Zealand) was used as the standard. The intra- and inter-assay coefficients of variation were 6.5% and 9.1% respectively. The minimal detectable dose was 0.03 ng/tube and the half-maximal displacement value in the assay was 0.6 ng/tube.

Growth hormone and insulin concentrations were measured using heterologous double antibody radioimmunoassays (RIA) described previously (Flux *et al.* 1984). The GH assay used bovine GH for iodination (USDA - bGH - I1, 3.2 IU/mg) and reference standards (USDA - bGH - B1, 1.9 IU/mg). Bovine insulin (Catalogue No. I - 1550, Lot No. 55F-0536, 23.4 IU/mg, Sigma Chemical Co.) was used for iodination and as the reference standard in insulin RIA. Intra- and inter- assay coefficients of variation for GH and insulin RIA were 8.6 and 13.2%, and 8.2 and 12.4%, respectively.

Placental lactogen concentrations in plasma were measured by homologous radioimmunoassay (Oliver *et al.* 1992) using recombinant oPL (Lot # M3RD78) as standard. The minimal detectable dose was 0.05 ng/tube, half-maximal displacement was achieved at 0.6 ng/tube and intra- and inter-assay coefficients of variation were 6.1%

and 8.7% respectively.

2.3.4 Mammary gland measurements

Dimensions of the mammary gland of each ewe were measured on days -1 and 8 (slaughter day) of treatment as described previously (Mellor & Murray, 1985). Three measurements were made from the posterior margin to the anterior margin of the udder, one down the midline and one on each side, parallel to the first measurement and immediately medial to each teat. The three measurements were then summed.

2.3.5 Slaughter procedures

Slaughter of the ewes, by captive bolt pistol and exsanguination, commenced at 0800 h on day 108 of pregnancy (8h after the last injection). The mammary gland was dissected off and trimmed of skin, fat and connective tissue before being weighed. Following removal of the mammary gland the abdominal cavity was opened and the uterus removed. A ligature was tied at the junction of the cervix and uterus, and the cervix, vagina and associated connective tissue removed. The uterus was then weighed (total uterine weight). The fetus was removed from the uterus and blood sampled (10 ml) by cardiac puncture. Fetal weight and sex were recorded. Caruncles were dissected from the uterus (after separation of fetal cotyledons), counted and their total weight recorded. The myoendometrium and fetal membranes (including cotyledons) were then weighed. Fluid weight was calculated by subtracting combined weights of the fetus, fetal membranes, myoendometrium and caruncles from the total uterine weight.

The liver, spleen, heart, kidneys and thymus of the ewes were dissected out, blotted dry and their fresh weights recorded (combined weights of bilateral organs). Carcasses were chilled and weighed.

2.3.6 Statistical analyses

Effects of treatment on parameters measured at a single time (liveweight, carcass weight, mammary gland (MG) dimension, trimmed MG weight, organ weights, weights of uterine components and fetal hormone concentrations) were analyzed by one-way analysis of variance. Data are expressed as least square means and standard errors for the three treatment groups (covariate-adjusted where appropriate). Covariate adjustment

was undertaken using the covariate (eg maternal age or weight) which accounted for the greatest proportion of residual variation (no covariate used where none was significant). Comparisons between pairs of treatments (saline, oPL, bGH) were made by orthogonal contrasts. Plasma hormone/metabolite concentrations on day 7 were covariate-adjusted for corresponding values on day -1. Metabolite/hormone concentrations measured on more than one occasion within a day were averaged. Prior to analysis, intakes of sheep nuts and lucerne chaff were converted to a total metabolisable energy (ME) intake based on assumed ME contents (per kg dry matter) of 9.9 MJ and 9.0 MJ, respectively (Ulyatt *et al.* 1980). One ewe (in the oPL group) was found at slaughter to be carrying twin fetuses. Data from this ewe and her fetuses were therefore deleted from all analyses. Statistical analyses were conducted using the computer package 'SAS'(1986).

2.4 RESULTS

2.4.1 Plasma metabolite and hormone concentrations

Maternal plasma concentrations of hormones and metabolites on day 7 of treatment are in Table 2.1. oPL treatment significantly ($P<0.05$) elevated circulating concentrations of oPL compared with those in saline-treated animals which in turn had higher ($P<0.05$) concentrations of oPL than the bGH group. Despite their contrasting effects on maternal circulating concentrations of oPL, both oPL and bGH treatment significantly depressed plasma concentrations of oPL in the fetus compared to those of fetuses from saline-treated ewes (saline-treated, 15.2±1.0 ng/ml; oPL-treated, 12.1±1.1 ng/ml; bGH-treated, 11.8±1.1 ng/ml, $P<0.05$).

bGH treatment increased the mean maternal concentrations of GH to levels approximately 9-fold ($P<0.001$) those observed in either saline- or oPL-treated animals. Consistent with the increased circulating concentrations of GH, plasma IGF-I concentrations in animals treated with bGH were increased ($P<0.001$) compared to saline-treated or oPL-treated ewes. bGH treatment of the pregnant ewes also increased plasma IGF-I concentrations in the fetuses (81.4±3.9 ng/ml) relative to those in fetuses of either the saline (71.0±3.9 ng/ml) or oPL (70.9±4.2 ng/ml) groups ($P<0.10$). Conversely, plasma IGF-II concentrations of the bGH-treated ewes were significantly ($P<0.001$) reduced relative to those of the other two groups. A corresponding reduction

in IGF-II concentrations was also apparent (P<0.10) in the fetuses of the bGH-treated ewes (956.4±59.3 ng/ml), compared with fetuses of saline- (1031.3±55.1 ng/ml) or oPL- (1100.8±60.2 ng/ml) treated ewes. Treatment of ewes with oPL had no significant effect on maternal or fetal plasma concentrations of IGF-I or IGF-II relative to saline treatment.

bGH treatment significantly (P<0.01) increased plasma insulin and glucose concentrations, while oPL treatment had no effect on either parameter. bGH treatment also increased plasma NEFA concentrations (P<0.10) and reduced creatinine concentrations (P<0.05) relative to those in the saline or oPL groups.

Table 2.1 Effects of saline (Sal.), bovine growth hormone (bGH) and ovine placental lactogen (oPL) on maternal circulating concentrations of hormones and metabolites on day 7 of treatment (mean±SE)

Parameter (unit)[d]	Treatment		
	Sal.	bGH	oPL
n	8	8	7
oPL (ng/ml)	84.6±6.6[b]	55.0±6.6[a]	127.2±9.1[c]
GH (ng/ml)	3.29±2.15[a]	27.1±2.12[b]	4.02±2.31[a]
IGF-I (ng/ml)	139.4±6.8[a]	341.0±6.8[b]	145.9±7.2[a]
IGF-II (ng/ml)	465.9±32.2[a]	315.2±32.3[b]	522.9±34.8[a]
Insulin (pg/ml)	195.4±248.4[a]	2128.6±259.1[b]	325.3±275.2[a]
Glucose (mM)	3.69±0.39[a]	5.68±0.39[b]	3.38±0.40[a]
NEFA (mEq/l)	0.13±0.03	0.22±0.03	0.13±0.03
Urea (mM)	6.69±0.36[a]	5.29±0.37[b]	6.65±0.37[a]
Creatinine (mM)	0.070±0.002[a]	0.065±0.002[b]	0.069±0.002[ab]

[abc] Means with different superscripts are significantly different (P<0.05)

[d] Adjusted to a common value on day -1 of treatment

2.4.2 Energy intake, body and organ weights

Average energy intakes, liveweights and carcass weights of the treated ewes are presented in Table 2.2. None of these parameters was significantly affected by treatment.

Neither bGH nor oPL caused a significant change in the weights of most maternal organs (Table 2.2). The only significant effect was observed with the spleen, oPL-treated ewes having lower spleen weights ($P<0.05$) than the saline-treated ewes while those of the bGH-treated ewes were intermediate. oPL treatment also marginally ($P<0.10$) depressed the weight of the heart relative to those of ewes in the other two groups.

Table 2.2 Effects of saline (Sal.),bovine growth hormone (bGH) and ovine placental lactogen (oPL) on energy intake, final liveweight, carcass weight and organ weights in pregnant ewes (mean±SE)

Parameter (unit)	Treatment		
	Sal.	bGH	oPL
n	8	8	7
Energy intake (MJ/day)[c]	18.5±1.2	17.4±1.2	18.1±1.2
Live weight (kg)	59.3±1.9	59.7±1.9	58.8±2.0
Carcass weight (kg)	27.4±1.5	29.1±1.5	27.4±1.6
Weight (g)			
Liver	1087.9±70.6	1177.5±67.5	1053.6±62.2
Spleen[d]	120.5±6.7[a]	113.5±6.6[ab]	98.5±7.2[b]
Heart	297.2±8.1	295.7±8.1	275.1±8.7
Kidney	162.0±6.6	152.8±6.6	156.0±7.1
Thymus[e]	182.1±17.3	204.4±17.5	183.2±18.5

[ab] Means with different superscripts are significantly different ($P<0.05$)

[c] Mean energy intake over 7 day treatment period covariate-adjusted to a common energy intake during the 5 days immediately prior to treatment

[d] Adjusted to a common age

[e] Adjusted to a common carcass weight

2.4.3 Mammary gland and uterine components

Mammary gland (MG) weights/dimensions and weights of uterine components measured at slaughter are shown in Table 2.3. There was a significant positive correlation (pooled across groups) between maternal circulating concentrations of oPL on day -1 of treatment and trimmed MG weight ($r=0.53$, $P<0.01$). However, there were no clear effects of treatment on MG dimension or trimmed MG weight.

Maternal plasma concentrations of oPL on day -1 of treatment were positively correlated (pooled across groups) with both total uterine weights ($r=0.47$, $P<0.05$) and fetal weights ($r=0.47$, $P<0.05$). Despite this, treatment with exogenous oPL had no significant effects on weights of any of the uterine components. In contrast, bGH treatment resulted in a significant ($P<0.05$) increase in the total weight of the gravid uterus. This effect did not reflect a difference in fetal weight. Rather, it was due mainly to significant ($P<0.05$) increases in weights of the fetal fluids, fetal membranes and myoendometrium. bGH-treated ewes also tended to have greater numbers of caruncles compared with those of oPL-treated ewes although the difference was only marginally significant ($P<0.10$).

Table 2.3 Effects of saline (Sal.),bovine growth hormone (bGH) and ovine placental lactogen (oPL) on mammary gland (MG) and uterine components in pregnant ewes (mean±SE)

Parameters (unit)	Treatment		
	Sal.	bGH	oPL
n	8	8	7
MG dimension (cm)[c]	41.7±1.2	39.8±1.2	39.5±1.2
Weight (g)			
Mammary gland	274.5±32.3	250.4±32.3	206.0±34.5
Gravid uterus	3984.9±385.5[a]	5260.3±385.5[b]	3697.1±412.1[a]
Fetus[d]	1804.5±135.8	1960.5±135.8	1727.3±147.4
Fluids	1312.0±248.4[a]	2289.6±248.4[b]	1255.1±265.6[a]
Fetal membranes	125.5±9.6[ab]	150.5±9.6[b]	111.6±10.3[a]
Myoendometrium	512.9±34.9[a]	620.8±34.9[b]	488.9±37.4[a]
Caruncles (total)	190.7±22.8	208.9±22.8	183.0±24.4
Caruncles (average)	2.15±0.3	2.39±0.3	2.31±0.3
No. of Caruncles	86.2±5.1	95.0±5.1	80.0±5.4

[ab] Means with different superscripts are significantly different ($P<0.05$)

[c] Adjusted to a common pre-treatment udder dimension

[d] Adjusted to a common sex

2.5 DISCUSSION

The objective of this study was to examine effects of recombinant oPL on maternal metabolism, mammary gland growth and uterine development/fetal growth in ewes. The limited supply of recombinant oPL available constrained the study to examining effects of oPL over a short period of time (7 days). However, treatment with recombinant bPL over the same period as used in this study has been shown previously to have significant

biological effects on both mammogenesis and lactogenesis in dairy heifers (Byatt *et al.* 1994). Single-bearing ewes were studied because they have lower circulating oPL concentrations than twin-bearing ewes (Gluckman *et al.* 1979*b*; Butler *et al.* 1981) and hence a presumed greater capacity to respond to exogenous oPL. Administration of oPL was undertaken during the period 101-107 d of pregnancy in order to elevate circulating oPL levels in treated ewes slightly in advance of the peak in circulating endogenous levels (Gluckman *et al.* 1979*b*; Taylor *et al.* 1980; Butler *et al.* 1981; Kappes *et al.* 1992). A third group of ewes was treated with recombinant bGH in order to compare the effects of bGH and oPL, particularly their relative effects on circulating IGF-I and IGF-II and metabolite concentrations, and on mammary gland development because there is some evidence that exogenous bGH stimulates mammary gland development in non-pregnant ruminants, including sheep (Johnsson *et al.* 1986).

The recombinant oPL preparation used in this study was more somatogenic than bGH in GH-deficient rodents (Singh *et al.* 1992). The same preparation of oPL, when injected twice daily at a dose of 0.10 mg/kg LW/day, increases the circulating insulin concentrations, growth rate and feed intake of milk-fed lambs (Chapter 4). Furthermore, this preparation induces insulin resistance in sheep when co-administered with bGH, whereas neither oPL nor bGH alone achieves this (Ogawa *et al.* 1995). Thus the preparation of oPL used here is known to be biologically active in sheep tissues, despite its limited effects in the present study.

Bovine PL (bPL) binds to somatotropic receptors in bovine liver (Wallace & Collier, 1984) and the administration of bPL to cows increases circulating IGF-I, but not IGF-II, concentrations (Byatt *et al.* 1992*b*). The administration of hPL to rats on a low-protein diet restored circulating IGF-I and IGF-II concentrations to those of well-fed pregnant rats (Pilistine *et al.* 1984) although elevation of IGF-I concentrations was not observed in non-pregnant rats on a normal diet treated with hPL (Chiang & Nicoll, 1992). Partially purified oPL has been reported to increase circulating IGF-I concentrations in hypophysectomized rats (Hurley *et al.* 1977), while the same batch of recombinant oPL used in this study significantly increased plasma IGF-I and growth rate in GH-deficient dwarf rats (Singh *et al.* 1992). However, Waters *et al.* (1985) found that passive immunization against oPL failed to alter plasma IGF-I concentrations in pregnant ewes. The present results are consistent with the latter study since they showed no effects of exogenous oPL on IGF-I or IGF-II concentrations, despite a significant effect of bGH

administered at the same dose. Thus, any effects of oPL in pregnancy are apparently not mediated via changes in circulating IGF-I or IGF-II concentrations. The inconsistencies in the aforementioned experiments also highlight the importance of conducting studies in homologous species.

Other differences between bGH and oPL were also demonstrated in this study. bGH treatment elevated maternal circulating glucose, NEFA and insulin concentrations and depressed urea and creatinine concentrations. In contrast, oPL treatment had no effects on these parameters. These results are inconsistent with an earlier report that infusion of oPL into non-pregnant ewes increased plasma concentrations of glucose, NEFA and urea (Thordarson *et al.* 1987). However, the preparation of oPL used by Thordarson *et al.* (1987) was not extensively purified. The earlier preparations may have been contaminated by other placental somatotropins, as shown in rat (Ogilivie *et al.* 1990) and human (MacLeod *et al.* 1991), which would account for some of these metabolic effects. When activity of endogenous oPL in pregnant ewes was altered by passive immunization against oPL, circulating concentrations of glucose and NEFA were not altered but insulin concentrations were increased (Waters *et al.* 1985). Thus, differences between the present study and some of the previous studies most likely reflect different preparations of oPL. Consistent with the present results, recent studies reported that recombinant bPL had no effects on circulating concentrations of glucose, NEFA and insulin, but decreased urea concentrations, in non-pregnant and pregnant cows (Byatt *et al.* 1992*b*).

It has been suggested that there are specific receptors for oPL (Freemark *et al.* 1987, 1988). However, recent studies question this interpretation. oPL and oGH binding show parallel changes in ontogeny in the sheep (Breier *et al.* 1994*a*). oPL binds to the ovine GH receptor expressed in COS cells (Breier *et al.* 1992). Detailed ligand binding and affinity cross-linking studies suggest that oPL and oGH bind to GH receptors in sheep liver (Breier *et al.* 1994*a*).

Similarly, immunoreactivity with monoclonal antibodies against the extracellular region of the GH receptor provides strong evidence that oPL binds to the GH receptor or a related protein (Breier *et al.* 1994*b*). Although oPL binds to the GH receptor with a 10-fold higher affinity than oGH (Breier *et al.* 1994*b*), the sequence of oPL (Colosi *et al.* 1989) does not allow GH receptor homodimerization. Thus, the differences in biological action of oPL and oGH might be a function either of qualitatively different

binding to the GH receptor or binding to a related protein.

bGH treatment stimulates mammogenesis in non-pregnant ruminants (Johnsson *et al.* 1986), even though it is well established that the concentrations of GH receptors in the mammary gland of ruminants are low (Akers, 1985). Thus, the actions of GH on the mammary gland may be indirect and mediated by the IGFs since IGF-I increases DNA synthesis in bovine mammary tissues *in vitro* (Baumrucker & Stemberger, 1989). bPL administration is also able to stimulate DNA synthesis in bovine mammary tissue maintained in athymic nude mice (Vega *et al.* 1989) which supports the view that PL may have stimulatory effects on mammary gland development in ruminants. Direct evidence of this is provided by a recent study (Byatt *et al.* 1994) in which treatment with recombinant bPL over a period of 7 days stimulated both mammogenesis and lactogenesis in steroid-primed dairy heifers. However, such effects were not apparent in the present study, where mammary gland development, as measured by dimension or trimmed weight of the mammary gland, was not affected by oPL treatment.

Inconsistencies between the present study and that of Byatt *et al.* (1994) may reflect a difference in experimental protocols, including doses of injected recombinant PL and the index of mammary gland development. For example, the dose of oPL used in the present study was 0.15 mg/kg LW/day which was much less than doses (between 0.25 and 0.50 mg/kg LW/day) used in the previous study (Byatt *et al.* 1994). Furthermore, in the present study, circulating concentrations of oPL at day -1 of treatment were positively correlated with trimmed MG weights. The present data, therefore, do not necessarily preclude a role for PL in mammary gland development.

Studies with pregnant ewes, including the present study, have shown that circulating maternal oPL concentrations are correlated with fetal weight (Taylor *et al.* 1980, Kappes *et al.* 1992). Such relationships between maternal oPL and fetal growth, if causal in nature, are most likely mediated via alterations in maternal metabolism (Thordarson *et al.* 1987) since there is no evidence that PL in maternal circulation can reach fetal circulation across the placental barrier (Kappes *et al.* 1992). In the present study, maternal oPL treatment had no effects on weights of any of the uterine components, whereas treatment with bGH resulted in a significant increase in the total uterine weight. This resulted from a marked (75%) increase in uterine fluid weights and, to a lesser extent, in weights of the fetal membranes (20%) and myoendometrium (19%). However, the increased fluid weight observed here could not be partitioned according to changes

in amniotic vs. allantoic fluid weights. The observation of an increased caruncle number in bGH-treated ewes is also consistent with a previous report that bGH treatment of pregnant ewes increases the number, but not the total weight, of placentomes (Stelwagen *et al.* 1991).

In the sheep alterations in nutrition in early and mid pregnancy can lead to alterations in placental size, primarily by altering the number of cotyledons. The sheep placenta is composed of 80-150 cotyledons which each individually implant on specialized uterine sites (caruncles). It appears that placental size can be affected by alteration in the number of cotyledons established in early pregnancy or retained in midpregnancy. The present data suggest that maternal hormone status either directly or indirectly affects these processes.

Measurement of circulating oPL concentrations in the ewes and their fetuses suggested that exogenous bGH treatment suppressed placental secretion or increased clearance of this hormone. The mechanism responsible for this effect is presently unknown. An effect of bGH on oPL secretion seems unlikely given that bGH treatment increased the weights of the fetal placenta, although weight and secretory capacity are not necessarily correlated. However, it is possible that bGH may have a direct effect on oPL clearance since the two hormones compete for membrane binding in liver (Chan *et al.* 1978; Emane *et al.* 1986; Breier *et al.* 1994*a,b*).

In conclusion, oPL and bGH administered to pregnant ewes were found to exhibit markedly different effects on circulating concentrations of oPL, IGF-I, IGF-II, insulin and metabolites, and on weights of certain maternal organs (spleen and heart) and components of the gravid uterus. These observations, together with the previous report that bGH and oPL exert differential effects on expression and circulating levels of IGFBP-2 (Klempt *et al.* 1993), suggest that oPL and bGH may exert their effects via distinct mechanisms. The present data do not support earlier suggestions (Adams *et al.* 1983) that oPL may act via the endocrine IGF system.

CHAPTER 3

RECOMBINANT OVINE PLACENTAL LACTOGEN AND BOVINE GROWTH HORMONE HAVE DIFFERENTIAL EFFECTS ON GALACTOPOIESIS IN EWES

bGH treatment increased circulating IGF-I concentrations and milk yields in ewes during established lactation, whereas administration of the same dose of oPL failed to demonstrate such galactopoietic effects.

3.1 ABSTRACT

The effects of recombinant ovine placental lactogen (oPL) and bovine growth hormone (bGH) on milk yield, milk composition and blood hormone and metabolite concentrations were compared in ewes during established lactation. Beginning on day 17 of lactation, ewes were treated by twice daily subcutaneous injection for 5 days with oPL (n=9) or bGH (n=10) at a dose of 0.10 mg/kg liveweight (LW)/day or with saline (n=10). Circulating concentrations of oPL were 24.6±1.6 ng/ml on day 5 in the oPL-treated animals, but undetectable in either saline- or bGH-treated groups. bGH treatment increased circulating concentrations of GH ($P<0.05$), insulin-like growth factor-I (IGF-I) ($P<0.001$) and non-esterified fatty acids ($P<0.05$), and decreased urea ($P<0.05$) concentrations relative to those in oPL- or saline-treated ewes. None of these parameters was affected by oPL treatment (vs saline treatment). Neither bGH nor oPL treatment had significant effects on plasma concentrations of insulin, glucose, and creatinine. bGH treatment, but not oPL treatment, increased yields of milk, fat and lactose ($P<0.05$). Weight of the mammary gland was significantly ($P<0.05$) increased by bGH, but not oPL, treatment. Despite the fact that oPL is a potent somatogen, it does not appear to exhibit the same galactopoietic activity as bGH in the lactating ewe.

3.2 INTRODUCTION

Ovine placental lactogen (oPL) is a member of the growth hormone (GH)/prolactin (PRL) family that is produced by the binucleate cells of the chorionic epithelium (Kappes *et al.* 1992). Sequencing studies have shown that oPL shares considerable homology with bovine PL (67 %) and ovine PRL (48-49 %), and a lesser homology with ovine and other species of GH (27-28 %) (Colosi *et al.* 1989; Warren *et al.* 1990*a*). Consistent with its PRL-like structure, oPL displays lactogenic activity. It binds to PRL receptors in the rabbit mammary gland and stimulates lactogenesis in mammary explants from the pseudopregnant rabbit (Servely *et al.* 1983) although such effects are much less marked in mammary explants from pregnant and lactating ewes (Servely *et al.* 1983; Emane *et al.* 1986). Despite the low homology with oGH, oPL also displays somatogenic activity. It binds to the GH receptor in the sheep liver (Chan *et al.* 1976, 1978; Emane *et al.* 1986; Freemark *et al.* 1986; Breier *et al.* 1994*a,b*) and is equipotent to bGH in stimulating ornithine decarboxylase activity (Butler *et al.* 1978) and amino acid transport (Freemark & Handwerger, 1982) in postnatal tissues. Administration of

partially purified oPL increased plasma concentrations of IGF-I in hypophysectomized rats (Hurley *et al.* 1977), while administration of recombinant oPL stimulates weight gain in growth hormone-deficient dwarf rats (Singh *et al.* 1992) and in intact lambs (Chapter 4).

A recent study with dairy cows demonstrated that administration of recombinant bPL increased milk production during mid- and late-lactation (Byatt *et al.* 1992*a*) although apparently by different mechanisms to bGH. However, there have been no studies conducted to examine the effects of exogenous oPL on milk production in the ewe. This study examined the effects on milk production and composition of recombinant oPL administered to ewes during established lactation and compared these with the effects of bGH.

3.3 MATERIALS AND METHODS

3.3.1 Animals and treatment

Thirty twin-rearing Border Leicester x Romney ewes aged between 2 and 5 years were used in this study. All were mated at the second oestrous cycle after progesterone synchronization and twin pregnancy was confirmed using ultrasound diagnosis at days 55 and 75 of pregnancy.

The ewes lambed at pasture over a 9 day period. They remained with their lambs until day 3 of lactation, when they were separated permanently from their lambs, housed in individual pens, and treated intramuscularly with 2.5 million units/ewe prophylactic penicillin (Leocillin, Leo Pharmaceutical Products, Denmark) to prevent mastitis. Over the next two weeks, commencing from the evening of their arrival in the barn, the ewes were accustomed to the milking regimen which consisted of twice daily machine milking (1600h and 0700h). Since the ewes had not been milked previously, each animal was injected intravenously with 1 IU oxytocin (TAD Pharmazeutisches Werk GMBH D-2190 Cuxhaven 1, West Germany) immediately prior to each milking to ensure complete milk removal. Each morning after milking, the morning milk was pooled with the previous evening's milk (stored at 4°C overnight) and the total daily yield recorded. The pooled milk was then mixed thoroughly and samples taken for fat, protein and lactose analyses.

After a two week adjustment period (day 17 of lactation) the ewes were divided into three groups balanced for age and pretreatment milk yield. One group (n=9) was injected

subcutaneously with recombinantly-derived oPL (0.10 mg/kg liveweight (LW)/day; Lot #M3RD78, Genentech, San Francisco, CA), one group (n=10) with recombinantly-derived bGH (0.10 mg/kg LW/day; Lot #7368C-69Q, American Cyanamid, Princeton, NJ), and the control group (n=10) with sterile physiological saline (0.036 ml/kg LW/day). The oPL was the same batch as that shown to be somatogenic in the GH-deficient dwarf rat (Singh *et al.* 1992) and intact lamb (Chapter 4). bGH was solubilized at a concentration of 2 mg/ml in carbonate buffered saline (pH 9.4) while the oPL was provided as a 2 mg/ml solution in phosphate buffered saline (pH 7.6). Injection volumes were calculated on the basis of liveweight measured at day -2 of treatment. Subcutaneous injections were alternated between the right and left sides of the neck and were administered twice daily, at 0800 and 1600h (half the daily dose at each time). Treatment continued for 5 days (ie from the pm milking of day 17 to the am milking of day 22 of lactation inclusive).

The ewes were fed a diet of *ad libitum* lucerne chaff plus 1500g/day of sheep nuts throughout the experiment. Fresh feed was offered at 1500h and feed intakes were recorded daily. Water was available *ad libitum.* The study was approved by the Massey University Animal Ethics Committee.

3.3.2 Blood sampling

Blood was collected from each ewe by jugular venipuncture at 1500, 0800 and 1100h on days -1 and 5 of treatment. Samples (8 ml) were withdrawn into vacutainers (Nipro Medical Industries, Japan) containing EDTA as the anticoagulant and immediately placed on ice. Within 20 min the samples were centrifuged at 3000 g and 4°C for 20 min. Plasma was harvested into duplicate vials and stored at -20°C for subsequent analysis.

3.3.3 Chemical analyses

Plasma concentrations of glucose, non-esterified fatty acids (NEFA), urea, creatinine, insulin, GH and PL were determined as described previously (Chapter 2). Plasma IGF-I was analyzed after acid-ethanol cryo-precipitation extraction with excess IGF-II added to the assay buffer to eliminate interference of residual IGF binding proteins (Breier *et al.* 1991).

Concentrations of fat, protein and lactose in milk were measured using a Milkoscan

104 semiautomatic infrared analyzer (A/S N. Foss Electric, Denmark). Milk samples were diluted with water prior to assay to bring fat concentration within the range of instrument calibration (for bovine milk).

3.3.4 Slaughter procedures

Slaughter of the ewes commenced at 1300h on day 22 of lactation (5h after the last injection). The ewes were milked out with oxytocin and then weighed, stunned with a captive bolt pistol and exsanguinated. The mammary gland was immediately dissected off and trimmed of skin, fat and connective tissue before being weighed. The possible effects of milk accumulation from the last milking to the slaughter time on weights of the trimmed mammary gland (MG) were corrected by subtracting the calculated accumulated milk volumes (based on the interval between last milking and slaughter, and the calculated milk secretion rate for the previous 24h) from weights of the trimmed mammary gland.

Immediately after removal of the mammary gland, the liver, spleen, lungs, heart, kidneys, pancreas, uterus and ovaries were dissected out, blotted dry and their wet weights recorded (combined weights of bilateral organs). Carcasses were weighed.

3.3.5 Statistical analyses

Data arising from single-time measurements on the animals (final liveweight, carcass weight, trimmed mammary gland weight, organ weights, milk yield and composition, energy intake) were subjected to one-way analysis of variance to test treatment effects. Covariate adjustment was undertaken using the covariate (eg age or weight) which accounted for the greatest proportion of residual variation (no covariate used where none was significant). Comparisons between pairs of treatments (saline, oPL, bGH) were made by Student's t-test. Data arising from repeated measurements on animals (metabolite/hormone concentrations) were subjected to multivariate (repeated measures) analysis of variance to test the effects of treatment, time (repeated factor) and their interaction. Plasma metabolite/hormone concentrations measured on more than one occasion within a day were averaged. Prior to analysis, intakes of sheep nuts and lucerne chaff were converted to a total metabolisable energy (ME) intake based on assumed ME contents (per kg dry matter) of 9.9 MJ and 9.0 MJ, respectively (Ulyatt *et al.* 1980). Statistical analyses were conducted using the computer package 'SAS' (SAS, 1986). All

data are presented as least square means and standard errors (covariate-adjusted where appropriate).

3.4 RESULTS

3.4.1 Plasma hormone and metabolite concentrations

Ewes treated with oPL had mean plasma PL concentrations of 24.6±1.6 ng/ml on day 5 of treatment but this hormone was undetectable in the plasma of saline- or bGH-treated ewes. Plasma concentrations of other hormones and metabolites are presented in Table 3.1. bGH treatment significantly increased plasma GH ($P<0.05$), IGF-I ($P<0.001$), and NEFA ($P<0.05$) concentrations (relative to saline treatment), whereas oPL treatment had no such effects. Conversely, bGH treatment tended to suppress plasma urea concentrations, while oPL treatment had the opposite effect. As a result, plasma urea concentrations of bGH-treated ewes were significantly ($P<0.05$) lower than those of oPL-treated ewes, with concentrations of saline-treated ewes being intermediate. Neither bGH nor oPL had effects on plasma insulin, glucose or creatinine concentrations.

Table 3.1 Effects of saline (Sal.), bovine growth hormone (bGH) and ovine placental lactogen (oPL) on plasma concentrations of hormones and metabolites in lactating ewes (mean±SE on day 5 of treatment covariate-adjusted for values on day -1)

Parameter (units)	Treatment		
	Sal.	bGH	oPL
n	10	10	9
GH (ng/ml)	7.92±0.8[a]	30.7±3.0[b]	12.3±3.2[a]
IGF-I (ng/ml)	119.7±9.4[a]	229.5±9.4[b]	130.8±10.0[a]
Insulin (pg/ml)	107.9±150.2	412.5±151.4	222.6±159.1
Glucose (mM)	3.01±0.10	3.09±0.10	3.01±0.11
NEFA (mEq/l)	0.27±0.09[a]	0.53±0.08[b]	0.38±0.09[a]
Urea (mM)	6.76±0.41[ab]	5.60±0.43[a]	7.21±0.44[b]
Creatinine (mM)	0.064±0.002	0.066±0.002	0.067±0.002

[ab] means with different superscripts are significantly different (P<0.05)

3.4.2 Milk yields, and contents and yields of milk constituents

Treatment effects on milk yields, and the contents and yields of major milk constituents, were analyzed using values derived over the last 3 days of treatment (Table 3.2). Milk yield following administration of bGH was significantly (P<0.05) increased over that of the controls (by 8 %), but was not influenced by treatment with oPL. There were no effects of treatment on milk composition (data not shown). Yields of fat (P<0.05), lactose (P<0.05) and protein (P<0.10) from bGH-treated ewes were increased relative to those from saline- or oPL-treated ewes.

Table 3.2 Effects of saline (Sal.), bovine growth hormone (bGH) and ovine placental lactogen (oPL) on yields of milk and of major milk constituents in lactating ewes (mean±SE, pooled over the last 3 days of treatment and covariate-adjusted for corresponding yields during the 5 days immediately prior to treatment)

Parameter (units)	Treatment		
	Sal.	bGH	oPL
n	10	10	9
Milk yield (g/day)	1546.3±35.9[a]	1664.9±35.8[b]	1495.8±37.2[a]
Fat yield (g/day)	99.0±2.9[a]	109.6±2.9[b]	92.1±3.0[a]
Protein yield (g/day)	90.1±3.0	96.4±2.8	88.7±2.9
Lactose yield (g/day)	85.8±2.3[a]	92.2±2.3[b]	80.4±2.4[a]

[ab] means with different superscripts are significantly different ($P<0.05$)

3.4.3 Energy intake, body and organ weights

Average energy intakes, liveweights and carcass weights of the treated ewes are presented in Table 3.3. Neither bGH nor oPL had significant effects on any of these parameters. As shown in Table 3.3, bGH treatment significantly ($P<0.05$) increased weights of the trimmed mammary gland (adjusted for residual milk) over those of saline or oPL treatments. bGH-treated ewes also had greater ($P<0.05$) liver weights than the oPL-treated ewes and marginally ($P<0.10$) heavier livers than saline-treated ewes. There were no other effects of treatment on organ weights.

Table 3.3 Effects of saline (Sal.), bovine growth hormone (bGH) and ovine placental lactogen (oPL) on energy intake, liveweight, carcass weight,trimmed mammary gland (MG) weight and liver weight in lactating ewes (mean±SE)

Parameter (units)	Treatment		
	Sal.	bGH	oPL
n	10	10	9
Energy intake (MJ ME/day)[c]	23.2±0.9	23.0±0.8	23.2±0.8
Liveweight (kg)[d]	54.7±1.2	54.1±1.2	55.8±1.2
Carcass weight (kg)[e]	23.5±0.8	23.1±0.8	22.7±0.8
Weight (g)			
Trimmed MG	765.1±38.5[a]	880.5±36.6[b]	772.0±36.6[a]
Liver	1061.7±42.1[ab]	1152.6±39.9[b]	963.3±40.0[a]

[ab] means with different superscripts are significantly different ($P<0.05$)

[c] mean energy intake over last 3 days of treatment covariate-adjusted to a common energy intake during the 5 days immediately prior to treatment

[d] adjusted to a common liveweight prior to treatment

[e] adjusted to a common slaughter liveweight

3.5 DISCUSSION

The objective of this study was to examine effects of recombinant oPL on milk production and composition in ewes during established lactation. The preparation of oPL used in this study is known to be highly somatogenic in both the GH-deficient rat (Singh *et al.* 1992) and the intact lamb (Chapter 4). Because of the limited supply of recombinant oPL available, the study was constrained to examining effects of oPL over a short period of time (5 days). It is well documented that exogenous bGH has galactopoietic effects in ruminants, including sheep, and that such effects are apparent after 4-5 days of treatment (Bauman & McCutcheon, 1986). Thus, a third group of ewes

was treated with recombinant bGH in order to compare the effects of bGH (the "positive control") and oPL on milk yield and the content of major milk constituents.

The failure of oPL to exhibit a galactopoietic effect is inconsistent with a previous report that recombinant bPL has galactopoietic effects in mid- and late-lactating dairy cows (Byatt *et al.* 1992*a*). Several factors could contribute to the different galactopoietic activities of bGH, oPL and, based on previous results (Byatt *et al.* 1992*a*), bPL. First, bGH treatment induced changes in circulating concentrations of NEFA and urea that were not observed with oPL treatment. Lipolytic effects are commonly observed in association with bGH-induced galactopoiesis but are not obligatory for galactopoietic responses since such responses have been reported in the absence of altered circulating metabolite concentrations (Bauman & McCutcheon, 1986). Furthermore, bPL achieves galactopoietic responses in lactating dairy cows without concomitant changes in the plasma concentrations of insulin, glucose or NEFA (Byatt *et al.* 1992*a*). Thus differences in circulating metabolite concentrations do not appear to account for the failure of exogenous oPL to stimulate milk yields.

Second, bGH treatment increased circulating IGF-I concentrations in the present study, while such effects were absent with oPL treatment. oPL and oGH bind with high affinity to hepatic membranes from pregnant and non-pregnant ewes (Chan *et al.* 1976, 1978; Emane *et al.* 1986; Breier *et al.* 1994*a,b*), and the same preparation of recombinant oPL used in this study stimulates circulating concentrations of IGF-I in GH-deficient dwarf rats (Singh *et al.* 1992). Furthermore, recombinant bPL has stimulatory effects on plasma concentrations of IGF-I in pregnant, non-pregnant and lactating cows (Byatt *et al.* 1992*a,b*). Thus, an effect of oPL on IGF-I levels might have been expected in this study. However, in pregnant ewes, administration of recombinant oPL had no effect on circulating concentrations of IGF-I, whereas bGH did increase IGF-I concentrations (Chapter 2). Waters *et al.* (1985) reported that plasma concentrations of IGF-I were not altered by passive immunization against endogenous oPL. Thus, results of the present study are consistent with the latter reports and suggest that oPL does not elevate IGF-I concentrations in sheep during short periods of treatment.

Studies *in vitro* (Gertler *et al.* 1983) and *in vivo* (McDowell *et al.* 1987*b*) suggest that bGH has no direct effects on the mammary gland of ruminants because of the very low number of GH receptors in mammary tissue (Akers, 1985). Thus, the actions of GH

on mammary gland appear to be indirect and are possibly mediated by the IGFs since IGF-I increases DNA synthesis of both ovine (Winder *et al.* 1989) and bovine (Baumrucker & Stemberger, 1989) mammary tissues *in vitro*, probably via the type-1 IGF receptor (Cullen *et al.* 1990). Significant increases in IGF-I concentrations in plasma with bGH, but not oPL, treatment in the present study are compatible with this hypothesis. The difference in milk yield response to bGH and oPL administration may be causally related to their differential effects on plasma IGF-I concentrations since systemic infusion of IGF-I in goats has been reported to induce a significant increase in milk yield (Prosser *et al.* 1990). However, the role of circulating IGF-I in galactopoiesis is uncertain in that the response to exogenous IGF-I is less than the response to a dose of bGH that achieves comparable concentrations of IGF-I in the blood (Davis *et al.* 1989*a*).

Third, it has been reported that there are marked differences between bGH and bPL in terms of effects on voluntary feed intake (Bauman *et al.* 1985; Peel *et al.* 1985). Feed intake increases within several days of recombinant bPL treatment (Byatt *et al.* 1992*a*), whereas such effects are not apparent until several weeks after the commencement of bGH treatment (Bauman *et al.* 1985; Peel *et al.* 1985). This increased feed intake appears to play an important role in supporting the galactopoietic effects of bPL in dairy cows since the increase in energy intake is highly correlated with increased energy secreted in milk by bPL-treated cows (Byatt *et al.* 1992*a*). Such increases in feed intake were also observed in rats treated with highly purified bPL (Byatt *et al.* 1991) and when milk-fed lambs were treated with recombinant oPL (Chapter 4). This observation suggests that the failure of oPL to increase voluntary feed intake in the present study was a consequence of the relatively short period of treatment. Thus, it is possible that the lack of an effect of oPL on milk yield in this study may have been partly due to the length of treatment. However, this would again indicate that bGH and oPL have different modes of action since bGH was capable of exerting a galactopoietic effect (in the absence of an effect on feed intake) within the period of treatment (5 days) used here. Furthermore, Byatt *et al.* (1992*a*) reported an effect of bPL on milk yield in cows with nine days of treatment but did not indicate whether the effect was apparent after the first 4-5 days as would be expected from bGH treatment (Bauman & McCutcheon, 1986).

In conclusion, the results presented here demonstrate that, unlike recombinant bGH,

oPL shows no acute galactopoietic activity in the ewe. The lack of a galactopoietic effect of oPL is associated with, and may be causally related to, its failure to induce elevated circulating IGF-I concentrations.

CHAPTER 4

THE EFFECTS OF RECOMBINANT OVINE PLACENTAL LACTOGEN (oPL) IN YOUNG LAMBS: COMPARISON WITH BOVINE GROWTH HORMONE PROVIDES EVIDENCE FOR A DISTINCT EFFECT OF oPL ON FOOD INTAKE

Chronic treatment with bGH had no significant effect on growth rate or feed intake in young lambs. In contrast, administration of the same dose of recombinant oPL stimulated both parameters.

4.1 ABSTRACT

The growth-promoting and metabolic effects of recombinant ovine placental lactogen (oPL) were compared with those of recombinant bovine growth hormone (bGH) in young lambs. Lambs were treated by twice daily subcutaneous injection with oPL (n=16) or bGH (n=16) at a dose of 0.1 mg/kg liveweight/day or with saline (n=16) for 21 days commencing on day 3 of life. Jugular blood samples were taken on days 0, 10 and 20 of treatment. Half the lambs in each group were slaughtered at 24 days, and the other half at 9 months, of age. Both bGH and oPL treatments induced small but significant ($P<0.05$) increases in circulating concentrations of insulin-like growth factor-I (IGF-I) on day 10 of treatment, but not on day 20. Neither treatment altered plasma concentrations of glucose, NEFA, urea or creatinine compared to those in saline-treated lambs. Relative to those of bGH- (0.24±0.01 kg/day) or saline- (0.25±0.01 kg/day) treated lambs, liveweight gains of oPL-treated lambs (0.28±0.01 kg/day) were significantly ($P<0.05$) increased during treatment and differences in liveweight were still apparent at 9 months age. Similarly, treatment with oPL, but not bGH, significantly ($P<0.01$) increased daily energy intake. It is concluded that placental lactogen and growth hormone do not have identical biological actions. While oPL is growth-promoting in young lambs, this effect may be mediated by stimulating voluntary feed intake rather than by elevating circulating concentrations of IGF-I.

4.2 INTRODUCTION

Ovine placental lactogen (oPL), a member of the growth hormone (GH)/prolactin (PRL) family, is a 198 amino acid protein (Colosi *et al.* 1989) produced by the binucleate cells of the chorionic epithelium (Kappes *et al.* 1992). Its physiological function in pregnancy is uncertain. Recent sequencing studies have shown that oPL has a higher degree of homology with ovine PRL(49 %) than with ovine or other species of GH (27-28 %) (Colosi *et al.* 1989; Warren *et al.* 1990*a*).

Despite its low homology with oGH, oPL displays somatogenic activity both *in vitro* and *in vivo*. It binds to oGH receptors in the sheep liver (Chan *et al.* 1978; Emane *et al.* 1986; Freemark *et al.* 1986; Breier *et al.* 1994*a,b*) or expressed in transfected COS cells (Adams *et al.* 1990; Breier *et al.* 1992). Administration of recombinant oPL also stimulates IGF-I production and weight gain in growth hormone-deficient dwarf rats

with a similar or superior potency to bGH (Singh *et al.* 1992). Similar effects have also been reported with highly purified bovine PL (bPL) (Byatt *et al.* 1991).

Although the above studies clearly demonstrate that oPL has somatogenic activities, these results have not been verified in a homologous system. This study examined the effects on body growth and composition, and metabolic parameters, of recombinant oPL administered to milk-fed infant lambs. A distinct effect on food intake was observed which may explain the growth-promoting actions of oPL and suggest a role for this hormone in determining maternal appetite during pregnancy.

4.3 MATERIALS AND METHODS

4.3.1 Animals and treatment

Forty-eight twin-born Coopworth x (Border Leicester x Romney) lambs were used in the trial. All lambs were born to ewes at pasture over a 9 day period in August (spring) and remained with their dams until day 3 of life to ensure adequate intakes of colostrum.

On day 3 of life the lambs were permanently separated from their dams, penned individually on slatted floors and assigned to one of three groups, each of 16 lambs, balanced for age and sex. The bam in which the lambs were held had an ambient temperature of 10-15 °C and natural photoperiod due to large windows at each end. Over the next three weeks, commencing on the evening of their arrival in the pens, one group of lambs was injected subcutaneously with recombinantly-derived oPL (0.10 mg/kg LW/day; Lot #M3RD78, provided by Dr R Vandlen, Genentech, South San Francisco), one group with recombinantly-derived bovine growth hormone (bGH) (0.10 mg/kg LW/day; Lot #7368c-69Q, provided by Dr IC Hart, American Cyanamid, Princeton, NJ) and the control group with sterile physiological saline (0.10 ml/kg LW/day). Subcutaneous injections were alternated between the right and left sides of the neck and administered twice daily, at 0800 and 1600h. Sites of injection were swabbed with 70% ethanol prior to injection. oPL was dissolved at a concentration of 1 mg/ml in phosphate buffered saline (pH 7.6) while bGH was dissolved as a 1 mg/ml solution in carbonate buffered saline (pH 9.4). The solutions for injection were prepared fresh each 3-7 days and held at 4°C. Injection volumes were adjusted to a new liveweight every 5 days

following reweighing of the animals.

During the three week treatment period the lambs were individually fed *ad libitum* a mixture of ovine and bovine milk (except during the first 3 days of the experiment when only ovine milk was offered). Milk (harvested from the dams of the lambs and from cows on the previous day and stored at 4°C overnight) was bottle-fed four times daily (at 0700, 1030, 1400 and 1700h) and was warmed to 38°C prior to each feeding. A few days after the commencement of bottle feeding some of the lambs developed mild scouring. Therefore, 1.5 ml Scourfix (1% sulphamethoxypyridazine, 0.2% trimethoprim plus kaolin and electrolytes, Vetchem Laboratories Ltd, East Tamaki, Auckland, NZ) was added to each 1 litre of milk and lambs were restricted to a maximum of 500 ml milk at the first feeding time (0700h). The ratio of ovine to bovine milk was 7:3 at days 6 and 7 of life, 6:4 at days 8 to 12, 5:5 at days 13 to 17, and 3:7 at days 18 to 23 of life. The composition of milk offered is presented in Table 4.1.

Table 4.1 Fat, protein and lactose content of milk offered during the experiment

	Composition (%)		
Day of life[a]	Fat	Protein	Lactose
3-5	7.92	5.66	5.42
6-7	5.54	4.38	5.34
8-12	4.94	4.40	5.31
13-17	4.84	4.38	5.04
18-23	4.80	4.28	5.08
24-59	3.58	3.37	4.90

[a] Lambs treated with saline, bGH or oPL on days 3 to 23 of life inclusive

After a three week treatment period (ie on day 24 of life) half of the animals from each treatment group (randomly selected on day 7 of treatment but balanced for sex) were slaughtered. The remaining lambs were offered *ad libitum* bovine milk for another

two weeks, to measure any carryover effects of treatment on voluntary feed intake. These lambs were then gradually weaned from the milk on to sheep nuts and lucerne chaff over a three week period (from days 38 to 59 of life) to facilitate early and gradual adaptation of the ruminal flora. The sheep nuts and lucerne chaff were offered *ad libitum* until day 59 of life. A mineral supplement (w/w 59% sodium chloride, 37% sodium sulphate, 4% sodium molybdate) was fed with the lucerne chaff at 2g/head every two days to counteract possible copper toxicity. From days 60 to 72 of life the lambs were offered *ad libitum* freshly cut white clover and lucerne chaff. Intakes were recorded at each feeding time and fresh water was available *ad libitum*. On day 72 of life the lambs were weaned on to clover-dominant pastures. All animals were in good health at the time of weaning. Lambs not slaughtered on day 24 were weighed at weekly intervals until day 59 of life, and thereafter on days 79, 156, 186, 201, 250 and 270 of life. The study was approved by the Massey University Animal Ethics Committee.

4.3.2 Blood sampling

Blood was taken from each lamb by jugular venipuncture at 0800, 1100 and 1500h on days 0, 10 and 20 of treatment (days 3, 13 and 23 of life). Samples (5 ml) were withdrawn into vacutainers (Nipro Medical Industries, Japan) containing EDTA as the anticoagulant and immediately placed on crushed ice. Within 20 min of collection the samples were centrifuged at 3000 g and 4°C for 20 min. Plasma was pipetted into duplicate vials and stored at -20°C for later analysis.

4.3.3 Organ and carcass measurements

Slaughter of half the lambs commenced at 1400h on day of 24 of life (7 h after the last injection), while the remaining lambs were slaughtered on day 270 of life. The lambs were weighed, stunned with a captive bolt pistol and exsanguinated. The skin, head, feet (between the carpals and metacarpals, and between the tarsals and metatarsals, of the fore- and hind-limbs, respectively) and viscera were removed. The liver, spleen, thymus, lungs, heart, kidneys and pancreas were dissected out, blotted dry and their fresh weights recorded (combined weights of bilateral organs).

Carcasses were chilled overnight (at 4°C) and their weight recorded. Measurements were then made of body length, maximum shoulder width, and maximum width of the

hindquarter (gigot width) (Moxham & Brownlie, 1976).

The carcasses were cut in half, the left side of each carcass frozen, sliced, and ground (Purchas & Beach, 1981), and representative samples of the homogenates freeze-dried (48 h) before being stored for later analysis (only for lambs slaughtered on day 24 of life). The right hind legs of all lambs (removed by a cut between the last and second to last lumbar vertebrae and perpendicular to the spine) were weighed and dissected into muscle, bone, and fat (Brown & William, 1979). The femur and humerus were weighed and their maximum length recorded (day 24 lambs only).

4.3.4 Wool growth measurements

On day 7 of treatment (day 10 of life) half of the lambs from each treatment group (those not to be slaughtered on day 24) had midside patches (over the last rib) cleared of wool to skin level with small animal clippers. On day 21 of treatment (day 24 of life) wool was harvested from these patches and the patch areas (approximately 10 cm square) measured with calipers (Mitutoyo, Japan). Harvested wool was weighed, scoured and conditioned to constant weight at 65% relative humidity, and rate of clean wool growth per unit area of skin calculated as described by Antram *et al.* (1991).

4.3.5 Chemical and hormonal analyses

Plasma concentrations of urea, creatinine, glucose, non-esterified fatty acids (NEFA), IGF-I, insulin, PL and GH were measured using the methods described previously (Chapter 2).

Nitrogen content of the carcass homogenate samples was determined by a macro-Kjedahl method using a Kjeltec Auto 1030 analyzer (Tecater A.B., Sweden). Fat was extracted from the samples by Soxhlet extraction for 8 h using petroleum ether (OMA, 1980).

4.3.6 Statistical analyses

Data arising from single-time measurements on the animals (organ weights, carcass weight, hind leg and carcass composition, wool growth) were subjected to one-way analysis of variance to test treatment effects within slaughter age. These parameters (other than wool growth, which was corrected for skin area) were adjusted to a common

carcass weight by covariance analysis. Data arising from repeated measurements on animals (liveweight, energy intake and metabolite/hormone concentrations) were subjected to multivariate (repeated measures) analysis of variance to test the effects of treatment, time (repeated factor) and their interaction. Plasma metabolite/hormone concentrations measured on more than one occasion within a day were averaged. Where appropriate, values of these parameters immediately prior to treatment were fitted as covariates in each case. Prior to analysis, intakes of milk were converted to total metabolisable energy (ME) intakes based on assumed ME contents of fat (39.2 MJ/kg), protein (24.4 MJ/kg) and lactose (16.5 MJ/kg), respectively (Holmes & Wilson, 1987). All data are expressed as least squares means and standard errors. Statistical analyses were conducted using the computer package 'SAS' (1986). One lamb in the oPL group died on day 32 of age so that there were only 7 lambs in the oPL group slaughtered on day 270 of age.

4.4 RESULTS

4.4.1 Liveweight gain, feed intake and feed conversion ratio

Liveweights of all lambs during treatment and of surviving lambs during the two-week period immediately post-treatment are shown in Figure 4.1. Liveweights of oPL-treated lambs diverged from those of the other treatment groups, as indicated by a significant ($P<0.01$) treatment x time interaction. As a result, final liveweights on day 21 of treatment (day 24 of life) of the oPL group were significantly ($P<0.05$) greater than those of the saline group. bGH treatment, in contrast, had no effects on liveweight gain. Average daily weight gain during the three weeks of treatment was significantly ($P<0.05$) greater in oPL-treated lambs (0.28±0.01 kg/day) than in saline-treated (0.25±0.01 kg/day) or bST-treated (0.24±0.01 kg/day) lambs. Such differences were also apparent ($P<0.05$) over the two week post-treatment period (saline, 0.19±0.01 kg/day; bGH, 0.17±0.01 kg/day; oPL, 0.25±0.02 kg/day, $P<0.01$).

The increased rate of gain observed in the oPL group was accompanied by an increased energy intake (Figure 4.1). Total energy intakes during treatment of oPL-treated lambs (159.6±4.6 MJ ME) were significantly ($P<0.05$) greater than those of saline-treated (144.9±4.6 MJ ME) or bST-treated (142.7±4.6 MJ ME) lambs. This

increased energy intake with oPL treatment led to feed energy/gain ratios similar to those with saline or bGH treatments (saline, 28.2±0.6 MJ ME/kg; bST, 28.2±0.6 MJ ME/kg; oPL, 27.7±0.6 MJ ME/kg). The elevated levels of feed intake in the oPL group were still apparent over the two week post-treatment period when lambs remained on an all-milk diet (saline, 8.44±0.42 MJ ME/day; bGH, 7.80±0.42 MJ ME/day; oPL, 9.93±0.45 MJ ME/day, $P<0.01$).

Liveweights of surviving lambs from day 45 of life (3 weeks post-treatment) to day 270 of life are depicted in Figure 4.2. oPL-treated lambs maintained their liveweight advantage throughout this period as indicated by a significant treatment effect over time ($P<0.05$).

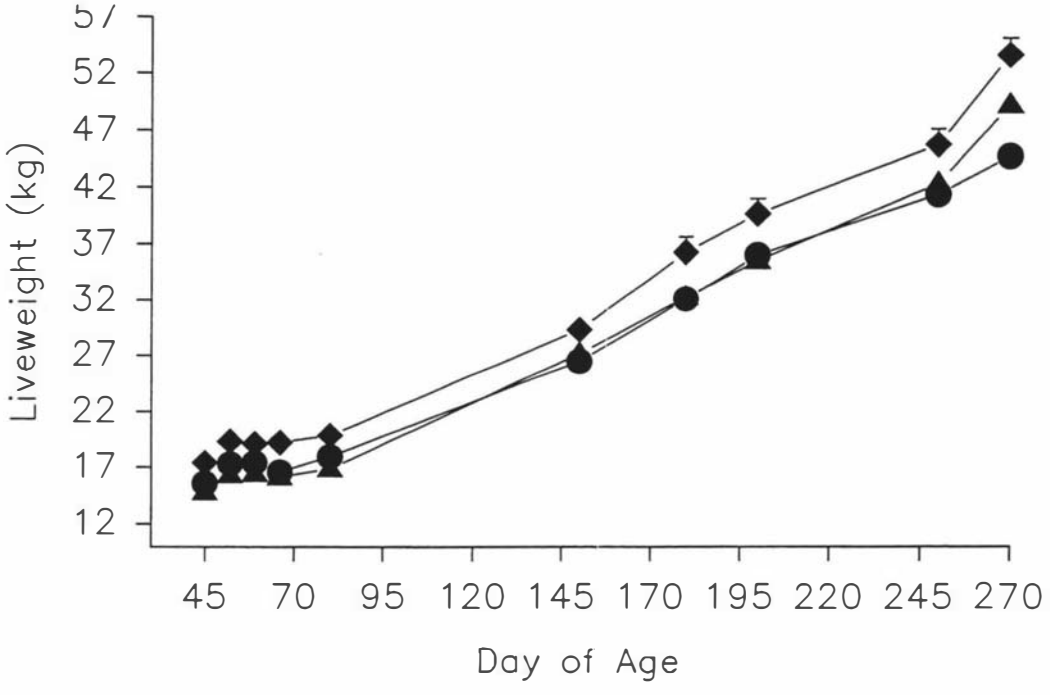

Figure 4.1 Effects of saline (●), bovine growth hormone (▲) and ovine placental lactogen (♦) on live weight and metabolisable energy intake of milk-fed lambs. Each point represents the means of 16 observations to day 21 and of 7 (oPL) or 8 (saline, bGH) observations from day 27. Treatment ceased on day 21. Vertical bars represent the pooled standard error of the mean. Treatment period is represented by solid lines, post-treatment period by broken lines.

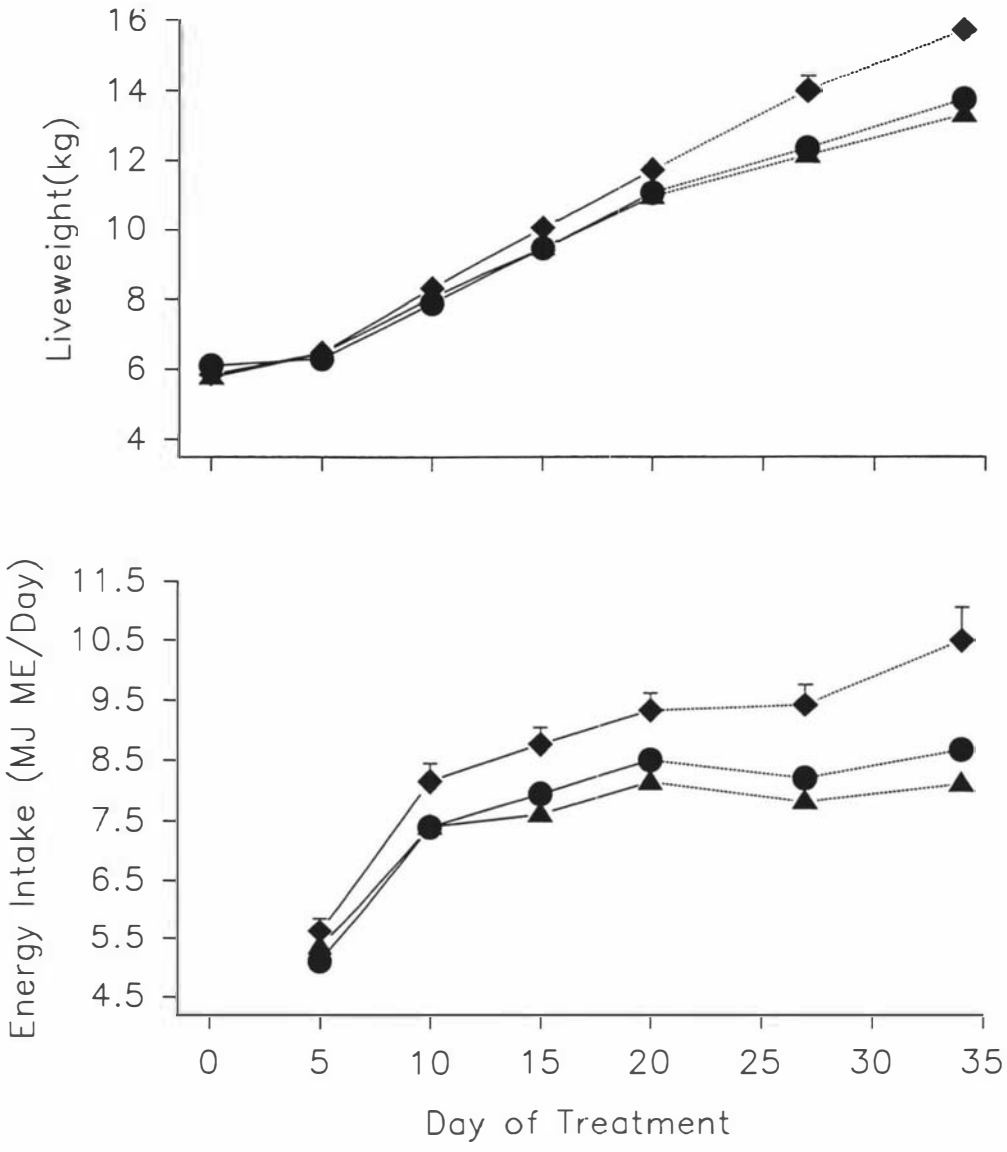

Figure 4.2 Liveweight of surviving lambs previously treated with saline (●, n=8), bovine growth hormone (▲, n=8) or ovine placental lactogen (♦, n=7) during days 45 to 270 of age (days 21 to 246 post-treatment). Vertical bars represent the pooled standard error of the mean.

4.4.2 Carcass parameters and hind leg composition

Neither bGH nor oPL had significant effects on measures of carcass size or hind leg composition of lambs slaughtered on day 24 of life (data not shown). However, carcass weights and shoulder widths at day 270 of lambs previously treated with oPL were significantly ($P<0.05$) greater than those of saline- or bGH-treated groups (carcass

weight: 25.7±0.9 vs 22.7±0.9 vs 22.9±0.8 kg; shoulder width: 215.2±5.0 vs 193.3±4.7 vs 201.1±4.1 mm). Again, neither bGH nor oPL had significant effects on hind leg parameters.

4.4.3 Organ weights

At day 24 of life, lung weights (adjusted to a common carcass weight) were significantly ($P<0.05$) lower in the bGH group (158.2±6.0g) than in the control group (177.9±6.0g), while those of the oPL-treated lambs (163.1±6.0g) were intermediate. In contrast, heart weights of bGH-treated lambs (81.9±2.4g) were marginally ($P<0.10$) greater than those of saline-treated lambs (75.8±2.4g), and significantly ($P<0.05$) greater than those of oPL-treated lambs (73.2±2.4g). All other differences were non-significant. Organ weights at day 270 of life were not influenced by previous treatment with oPL or bGH.

4.4.4 Plasma metabolite and hormone concentrations

Concentrations of oPL in plasma were below the assay detection limit in the bGH- and saline-treated groups, whereas oPL treatment increased circulating concentrations of oPL from undetectable levels at day 0 of treatment to (daily average) 15.7±0.6 ng/ml at day 10 of treatment with no further change at day 20 of treatment (13.4±0.6 ng/ml). There was marked variation in plasma oPL concentrations throughout the day. Thus, on day 10, mean concentrations were 0.6±0.2 ng/ml at 0800h, 38.9±1.5 ng/ml at 1100h and 6.9±2.3 ng/ml at 1500h. Plasma concentrations of GH and IGF-I during the study are depicted in Figure 4.3. Circulating concentrations of GH increased progressively during administration of bGH, while those of both the saline and oPL groups declined slightly or remained stable. The divergence of plasma GH concentrations between the bGH-treated lambs and the saline- or oPL-treated lambs resulted in a significant ($P<0.01$) treatment x time interaction. Plasma GH concentrations were, on day 10, 5.2±1.6 ng/ml at 0800h, 31.3±4.7 ng/ml at 1100h and 9.0±2.3 ng/ml at 1500h in the bGH-treated group. Treatment of lambs with bGH or oPL produced a small but significant ($P<0.05$) elevation of circulating IGF-I concentrations on day 10 of treatment but no significant effect on day 20 (Figure 4.3).

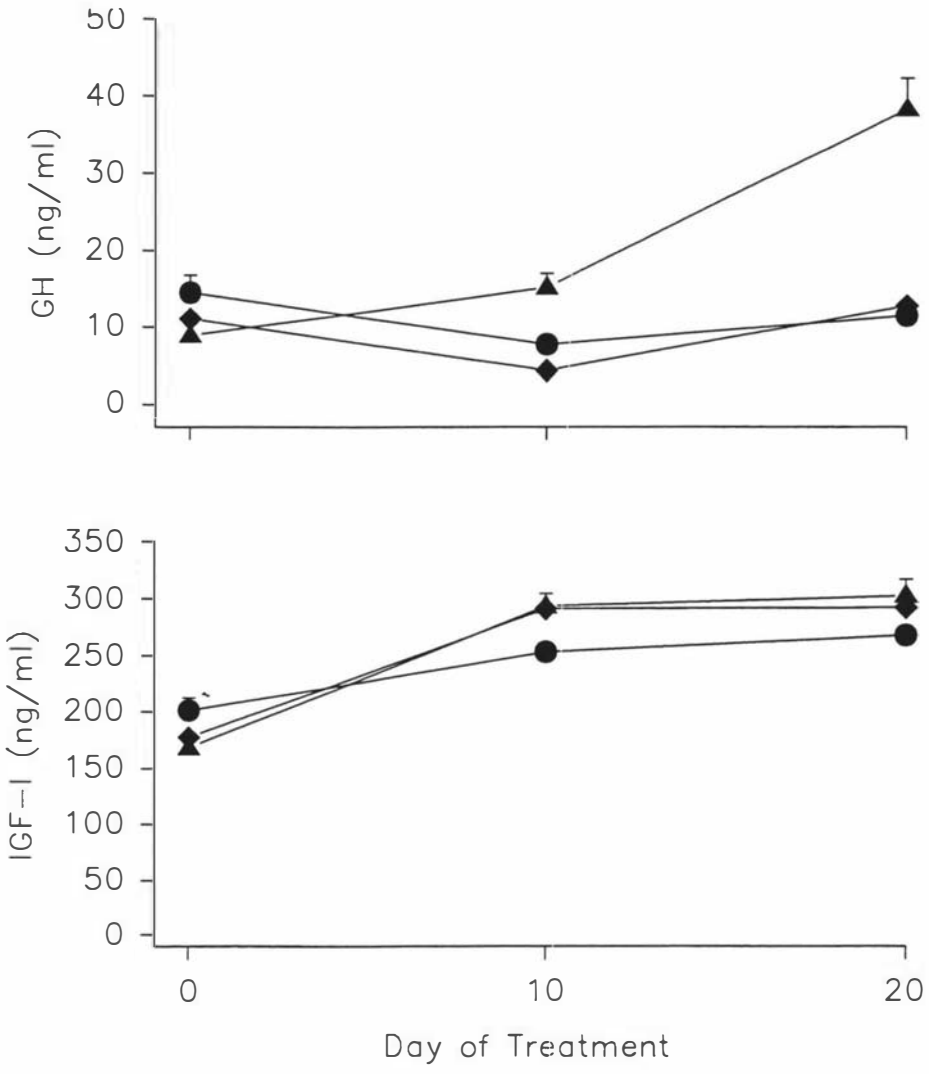

Figure 4.3 Effects of saline (●), bovine growth hormone (▲) and ovine placental lactogen (◆) on plasma concentrations of growth hormone and IGF-I in lambs. Each point represents the mean of 16 observations per group. Vertical bars represent the pooled standard error of the mean. Means for day 10 and day 20 are covariate-adjusted to a common pretreatment mean (day 0).

Mean plasma insulin concentrations on day 0, 10 and 20 of treatment are presented in Figure 4.4. Insulin concentrations following administration of bGH were greatly increased from 328.6±68.3 pg/ml on day 0 of treatment to 2160.7±317.1 pg/ml on day 10 of treatment, and to 1178.1±241.8 pg/ml on day 20 of treatment. Similarly, oPL treatment increased the mean concentrations of insulin to levels (2589.5±327.1 pg/ml

and 1321.9±241.8 pg/ml on days 10 and 20 of treatment, respectively) approximately twice those observed in saline-treated animals. Because of large variation within groups, however, differences in plasma insulin concentration between the bGH or oPL group and the saline group were only marginally ($P<0.10$) significant except on day 10 of treatment, when animals treated with oPL had significantly ($P<0.05$) greater plasma concentrations of insulin than those treated with saline. Neither bGH nor oPL had significant effects on plasma glucose or NEFA concentrations (Figure 4.4).

Neither bGH nor oPL treatment had effects on plasma urea concentrations (Figure 4.4) although bGH treatment marginally ($P<0.10$) decreased circulating concentrations of urea compared to those in the oPL treatment group. In contrast, bGH treatment increased plasma creatinine concentrations at day 10 and 20 of treatment relative to those of oPL treatment although such differences were, again, only marginally ($P<0.10$) significant (Figure 4.4).

4.4.5 Wool production

Neither bGH nor oPL had a significant effect on the rate of clean wool growth per unit area of skin during days 7-21 of treatment (saline, 2.77±0.21 mg/cm^2/day; bGH, 2.84±0.21 mg/cm^2/day; oPL, 2.78±0.21 mg/cm^2/day).

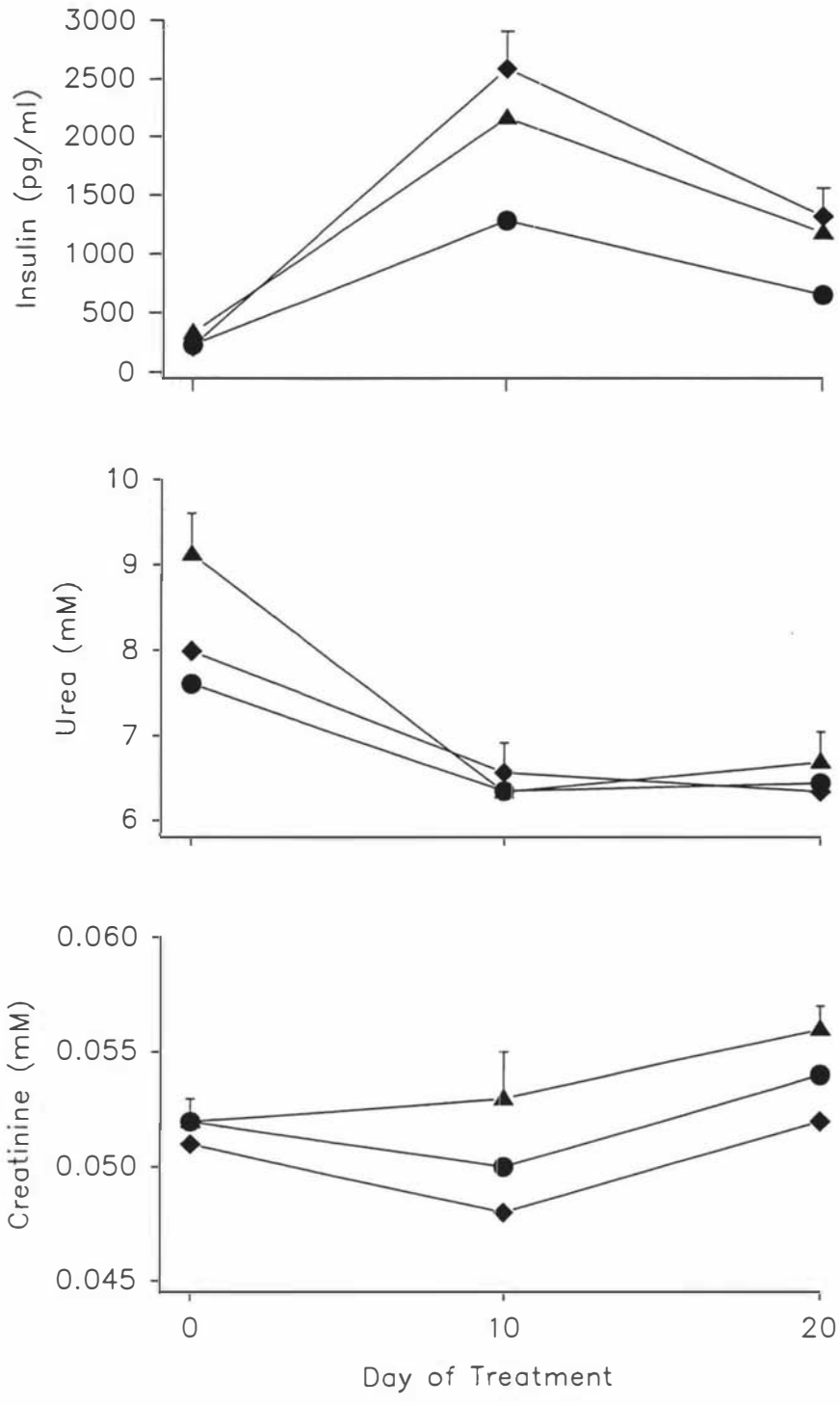

Figure 4.4 Effects of saline (●), bovine growth hormone (▲) and ovine placental lactogen (♦) on plasma concentrations of insulin, urea and creatinine in lambs. Each point represents the mean of 16 observations per group. Vertical bars represent the pooled standard error of the mean. Means for day 10 and day 20 are covariate-adjusted to a common pretreatment mean (day 0).

4.5 DISCUSSION

This is the first study to demonstrate a growth-promoting effect of oPL in the homologous species, and it provides further evidence that oPL and bGH act via different mechanisms.

oPL treatment significantly increased the live weights and body size of lambs from day 10 after commencement of treatment, and the greater liveweights of oPL-treated lambs were still apparent at day 270 of life (250 days after termination of treatment). Organ weights and body composition (at a common carcass weight) of oPL-treated lambs were little different to those of saline- or bGH-treated lambs, indicating that normal growth proportions were maintained. The basis of the persistent effect on body weight after oPL treatment in the neonatal period is not clear. It may suggest that appropriate neonatal therapy can reprogramme growth control mechanisms and this might suggest a new approach to the manipulation of farm animal growth.

In the present study GH had no significant effect on weight gain or body composition, and only a small effect on IGF-I levels, although it was shown that the same preparation of bGH is biologically active (galactopoietic) in the ewe (Chapter 3) and in the GH-deficient rat (Singh *et al.* 1992). The lack of an effect on growth is consistent with a previous study of GH treatment in neonatal lambs (Sun *et al.* 1992), although Pell *et al.* (1990) showed that in 9 week old lambs the same dose of bGH as used in this study was clearly somatogenic. Hepatic GH receptor numbers increase in lambs during the first few days after birth (Gluckman *et al.* 1983), implying the onset of GH-dependent growth, but it may be that maximal responsiveness to GH depends on maturation of postreceptor mechanisms. For example, defective postreceptor mechanisms have been implicated in the GH resistance observed on refeeding after starvation (Maiter *et al.* 1989).

Treatment of young lambs with recombinant oPL significantly increased circulating concentrations of insulin on day 10 of treatment. These elevated concentrations of insulin, but without a concomitant increase in plasma concentrations of glucose, may indicate that oPL has a direct effect on insulin secretion. Stimulatory effects of a crude oPL preparation on insulin secretion have also been reported in pregnant and non-pregnant sheep (Handwerger *et al.* 1976). These *in vivo* results are further supported by recent *in vitro* studies in which PL was shown to enhance ß-cell division and insulin

secretion in rat, mouse, and human islets (Brelje *et al.* 1993). Such increases in plasma insulin concentrations may be crucial during pregnancy, when profound alterations in maternal metabolism occur to accommodate the increasing nutrient demands of the fetus(es).

It is well documented that bGH treatment increases voluntary feed intake in sheep and cattle, but only after several weeks of continuous treatment (Bauman *et al.* 1985; Peel *et al.* 1985; Sandles *et al.* 1988). In contrast, administration of recombinant bPL stimulates feed intake in dairy cattle within 9 days after commencement of treatment (Byatt *et al.* 1992*a*). Such acute increases in feed intake have also been observed in rats treated with highly purified bPL (Byatt *et al.* 1991), indicating that there are marked differences between bGH and bPL in their effects on voluntary feed intake. The present results show that treatment with oPL, but not bGH, significantly increased feed intake in milk-fed lambs from 10 days after commencement of treatment. Thus, the present data are consistent with previous reports and suggest that ruminant PLs stimulate energy intake. It has been suggested previously that the higher feed intakes of bPL-treated rats are partly mediated through the PRL receptor (Byatt *et al.* 1991) since administration of oPRL but not bGH stimulates feed intake in mature female rats (Gerardo-Gettens *et al.* 1989).

The mechanism by which oPL stimulates body growth is unclear. IGF-I is considered to be an important mediator of the somatogenic effects of GH, particularly during the postnatal and adolescent growth periods (Daughaday & Rotwein, 1989; Sara & Hall, 1990). Recombinant IGF-I has also been shown to have growth-promoting effects in GH-deficient animals (Robinson & Clark, 1989). Since oPL treatment in the present study produced a small but significant increase in circulating concentrations of IGF-I (though only at day 10 of treatment), it is tempting to suggest that the growth-promoting effects of oPL are partly mediated by IGF-I. However, administration of bGH at the same dose elevated circulating concentrations of IGF-I to the same extent as oPL treatment, but failed to have significant effects on either body weight or body composition. Pell *et al.* (1987) also reported that, in one experiment, exogenous bGH had no effects on growth rate or lean tissue content of lambs, despite significantly increasing circulating concentrations of IGF-I. These results strongly indicate that increasing the circulating concentration of IGF-I does not necessarily stimulate growth

rate in animals with an intact somatotropic axis. This suggestion is further supported by the recent observation that administration of recombinant of IGF-I over an 8 week period had only minor growth-promoting effects in well-fed yearling sheep (Cottam *et al.* 1992). It therefore seems unlikely that the somatogenic effects of oPL observed in this study can be accounted for by the modest increases in circulating concentrations of IGF-I associated with oPL (and bGH) treatment. Effects of IGF-I treatment on body growth are examined further in Chapter 5.

Alternatively, the somatogenic effects of oPL may be mediated via changes in voluntary feed intake. Such a possibility was first raised by Byatt *et al.* (1991). In that study, highly purified bPL administered to female rats at four dose levels stimulated feed consumption and caused significant increases in weight gain. Although these increases in weight gain were generally associated with increases in plasma IGF-I concentrations, weight gain and feed consumption were stimulated by the highest dose of bPL without a concomitant increase in plasma concentrations of IGF-I. This finding, and the present results, strongly suggest that an increased feed intake plays an important role in mediating the somatogenic effects of PL independent of effects on IGF-I status.

Finally, these observations provide a possible a possible explanation of the biological role of oPL in pregnancy. Maternal oPL levels rise in late gestation in proportion to the increase in placental mass (Gluckman *et al.* 1979*a*) but the biological role of PL in pregnancy remains speculative. It has been suggested that PL may alter maternal metabolism to favour glucose utilization by the fetus (Kaplan & Grumbach, 1974). Appetite increases in late gestation, presumably to provide for the increased demands of placental metabolism and fetal growth. In species such as the sheep, late gestation occurs at the time of year when appetence and food intake are suppressed, perhaps because of seasonal changes in lactogenic hormones (Short, 1991). Placental production of an appetence-stimulating hormone would provide a mechanism of ensuring that appetite is maintained to support fetal growth and to give a competitive advantage over non-pregnant animals in a herd situation. Further, the persistent increase in appetence extending beyond the withdrawal of oPL in the present study may reflect a mechanism by which food intake is maintained during lactation.

CHAPTER 5

RESPONSES OF YOUNG ENERGY-RESTRICTED SHEEP TO CHRONICALLY ADMINISTERED IGF-I: EVIDENCE THAT IGF-I SUPPRESSES THE HEPATIC GROWTH HORMONE RECEPTOR

Chronic treatment with IGF-I failed to alter growth rates in energy-restricted sheep, but decreased circulating concentrations of GH and hepatic GHR numbers.

5.1 ABSTRACT

It has been shown previously that chronic administration (8 weeks) of IGF-I has little growth promoting effect in well-fed sheep. The aim of this study was to investigate the anabolic effects of IGF-I in energy restricted conditions in which circulating concentrations of IGF-I in control animals were expected to be low. Young castrate male sheep were offered chaffed lucerne at a rate equivalent to 110% maintenance and were treated by 3x daily sc injection for either 8 or 12 weeks with recombinant human IGF-I (150 ug/kg LW/day) or saline in a 2 x 2 factorial design (8 animals per cell). IGF-I treatment significantly increased plasma concentrations of IGF-I but reduced plasma concentrations of IGF-II, GH, urea and creatinine. Treatment with IGF-I also decreased ($P<0.1$) GH secretion in response to a GRF load, but significantly ($P<0.05$) increased NEFA response to an epinephrine load. The reduction in circulating GH levels was accompanied by a suppression of ^{125}I-oGH binding to hepatic microsomal membranes. This effect, if apparent in other tissues, may act as a feedback mechanism to limit the local synthesis of IGF-I and could explain why IGF-I treatment had little effect on growth rate of the sheep although it did increase nitrogen digestibility of the feed consumed, and decrease fat content of the hind leg. It also differentially promoted growth of the spleen, thymus and mandibular salivary gland, and increased blood counts of eosinophils. It is concluded that IGF-I does not have marked effects on growth rate or body composition in sheep fed a near-maintenance diet. Possible reasons include the associated suppression of GH secretion and action, which limits the ability of treated animals to repartition absorbed nutrients.

5.2 INTRODUCTION

Exogenous IGF-I has been shown to promote growth in hypophysectomized animals or strains that are genetically deficient in growth hormone (GH) (van Buul-Offers *et al.* 1986; Guler *et al.* 1988; Skottner *et al.* 1989; Pell and Bates, 1992; Butler *et al.* 1994). However, it remains unclear whether this extends to animals with an intact somatotrophic axis. A recent study from this laboratory showed that administration of IGF-I over an 8-week period had virtually no growth-promoting effects in well fed sheep (Cottam *et al.* 1992), an observation supported by other studies (Guler *et al.* 1989*a*; McGuinness and Cogburn, 1991; Tixier-Boichard *et al.* 1992). However, Schalch *et al.*

(1989) reported that infusion of IGF-I for 7 days had growth-promoting effects in energy-restricted normal rats, but not in *ad libitum* fed rats. Similarly, stimulation of growth and nitrogen retention with IGF-I administration was greater during malnutrition in rats (Yang *et al.* 1990).

These results suggest that the failure of IGF-I to promote growth in well fed animals may be due to the fact that they have higher circulating concentrations of endogenous IGF-I than malnourished animals. This study was therefore designed to evaluate whether administration of IGF-I had anabolic actions in energy-restricted animals, whose circulating concentrations of IGF-I were expected to be low, using an experimental protocol similar to that in our previous study (Cottam *et al.* 1992). It has been also demonstrated from previous studies (Jacob *et al.* 1989; Zenobi *et al.* 1992; Hussain *et al.* 1994) that treatment with IGF-I alters sensitivity of peripheral tissues to insulin. Therefore, this study also examined direct effects of chronic IGF-I treatment on responsiveness to metabolic challenges and determined whether such treatment altered GH secretion or responsiveness.

5.3 MATERIALS AND METHODS

5.3.1 Animals and treatment

Thirty-two Border Leicester x Romney castrate male sheep aged 8 months at commencement of the trial were used. The sheep were housed in individual pens and offered chaffed lucerne hay at a rate calculated to be equivalent to 110 % maintenance. The maintenance requirement was calculated as 0.55 megajoules of metabolisable energy (MJ ME)/$kg^{0.75}$ (Holmes & Wilson, 1987) and feed was assumed to contain 9.1 MJ ME/kg dry matter (DM) at 88.5% DM (Allam *et al.* 1983). Feed was offered and intake was recorded by measuring refusals at 1500h daily. On alternate days, 2g of a mineral supplement (59% sodium chloride, 37% sodium sulphate, 4% sodium molybdate w/w) was fed with the lucerne hay to counteract possible copper toxicity. Animals were weighed at two-week intervals throughout the study and feed allowance was recalculated accordingly. Fresh water was available *ad libitum.*

After a two-week adjustment period, the sheep were randomly divided into four equal groups representing two treatments (IGF-I treated vs control) and two treatment

periods (8 week or 12 week) in a 2 x 2 factorial design with 8 animals per cell. The 8 week treatment period was designed to parallel our previous study with well fed sheep (Cottam *et al.* 1992) and the 12 week period to give greater sensitivity in the detection of live weight (LW) and other changes. During the treatment period, half the animals were treated with recombinantly-derived human IGF-I (150 ug/kg LW/day; Ciba Geigy, Summit, N.J) and half with saline (0.025 ml/kg LW/day). Injections were made subcutaneously in the neck, on alternate sides, three times daily (0830, 1600 and 2100h). The IGF-I was dissolved in sterile pyrogen-free saline at a concentration of 2 mg/ml. The solution for injection was prepared fresh each 7 days. Injection volumes were adjusted to the new liveweight every two weeks following reweighing of the animals.

On treatment day 32 (8-week group) or 60 (12-week group) the sheep were transferred from the pens to metabolism crates for the determination of nitrogen balance. Following a 7-day adjustment period in the metabolism crates, faeces and urine were collected daily at 1700h for the next 10 days (ie days 40-49 and 68-75 of treatment respectively). Daily faecal outputs were recorded and samples of the faeces (10% of daily output) bulked in plastic bags which were sealed and frozen (- 20°C) for later analysis. Urine was separated from faeces via mesh trays under the crates and collected into buckets containing 80 ml of 50% H_2SO_4 per day to acidify the urine and prevent loss of nitrogen by volatilization. Daily urine volume was measured and samples of the urine (5% of daily output) were bulked in plastic bottles for each of the 10 days and frozen (- 20°C). Samples of the feed (about 200 g) were collected daily and frozen for chemical analysis.

On treatment day 49 (8-week group) or 77 (12-week group), jugular cannulae (14G, Batch 0851E, Critikin, Tokyo, Japan) were inserted under local anaesthesia (Lignavet, Batch PO37/138, HMC Ltd, U. K.). Immediately after cannulation, prophylactic antibiotic (Streptopen, Lot 533XH, Pitman-Moore Animal Health Ltd, Palmerston North, N. Z.) was administered by i/m injection. Over the next 4 days animals were subjected to intravenous metabolic challenges of (in sequence) glucose, insulin, epinephrine and growth hormone-releasing factor (hpGRF1-44). To facilitate administration of metabolic challenges, the animals within each treatment group were divided into two blocks (4 control and 4 IGF-I treated in each block) with block A receiving challenges at 0900h (1h after the IGF-I injection) and block B at 1500h (7h after IGF-I injection). Details

of the challenges (dose, concentration and sources) are presented in Table 5.1. Glucose, epinephrine and GRF were dissolved in sterile physiological saline while the insulin was dissolved in saline containing 3% bovine serum albumin (BSA). Challenges were administered via the jugular cannulae and were followed by 5 ml of sterile saline to flush the cannulae. Blood samples were withdrawn at -15, -10, -5, 2.5, 5, 10, 20, 40 and 60 minutes relative to the time of injection. Between blood samplings the patency of the cannulae was maintained with 0.9% saline containing 100 IU/ml sodium heparin (New Zealand Pharmaceuticals Ltd, Linton, N. Z.) and 0.4 ml/l oxytetracycline (Terramycine Q-110, Pfizer Laboratories, Auckland, N. Z.).

Table 5.1 Metabolic challenges

Challenge	Concentration	Dose (/kg/LW)	Source
Glucose	.4 g/ml	.17g	National Dairy Association Palmerston North, New Zealand
Insulin	.2 mg/ml	.01 mg	Phoenix Chemicals Ltd, Christchurch, New Zealand
Epinephrine	.1 ug/ml	1.0 ug	David Bull Laboratories, Victoria, Australia
hGRF1-44	15 ug/ml	1.0 ug	Peninsula Laboratories Inc., Belmont, California

5.3.2 Blood sampling

Blood samples were taken by jugular venipuncture at 0800 and 1300h on day -1 of treatment and at two-weekly intervals thereafter. Additional samples were taken at the beginning and end of treatment (days -1 and 56 or 84 of treatment) to monitor haematological profiles. Samples (10 ml) were withdrawn into vacutainers containing EDTA as the anticoagulant. During the metabolic challenges samples were withdrawn via the indwelling jugular cannulae and transferred to centrifuge tubes containing 0.1 ml

of 35 % (w/v) sodium citrate as the anticoagulant. All samples were kept on ice and within one hour were centrifuged at 2500g and 4°C for 20 minutes. Plasma was then pipetted off into duplicate vials and stored at -20°C until subsequent analyses.

5.3.3 Slaughter procedures

Slaughter of the (unfasted) sheep by captive bolt and exsanguination commenced at 1000h on day 57 or day 85 of treatment. The skin, head, feet and viscera were removed. The liver, spleen, kidneys, lungs, pancreas, heart, thymus, thyroid glands and salivary glands (parotid and mandibular) were dissected out, blotted dry and their fresh weights recorded. Samples of liver (only from the 8 week group) were immediately frozen on dry ice and then kept at -20°C for measurement of hepatic binding of oGH and oPL. Carcasses were chilled overnight (at 4°C) and their weight recorded. Measurements were then made of body length, maximum shoulder width, and maximum width of the hindquarter (gigot width) (Moxham & Brownlie, 1976). The right hind leg (removed by a cut between the last and second to last lumbar vertebrae and perpendicular to the spine) was weighed and dissected into muscle, bone, and fat (Brown & Williams, 1979). The femur and tibia were weighed and their maximum length recorded.

5.3.4 Chemical and cellular analyses

Plasma concentrations of urea, creatinine, glucose, non-esterified fatty acids (NEFA), IGF-I, IGF-II (measured only at the start and end of each period), insulin and GH were measured using the methods described previously (Chapter 2). IGF-I was measured by radioimmunoassay after extraction of the plasma by acid-ethanol extraction with cryo-precipitation (Breier *et al.* 1991) and addition of excess IGF-II to eliminate interference of residual IGFBPs in the assay as previously validated for ovine plasma. The antibody (878/4) has a cross reactivity with IGF-II of <0.01 % (Breier *et al.* 1994*a*).

Leucocytes, haemoglobin, packed cell volume (PCV) and mean corpuscular haemoglobin concentration (MCHC) were determined using a Cobas Counter (Cobas Minos Vet Analyzer, Roche Products (NZ) Ltd). Leucocytes were enumerated in 100-cell differential counts of blood smears stained with Leishmans stain.

The nitrogen content of feed, faeces and urine samples was measured using a macro-Kjeltec Auto 1030 analyzer (Tecator A.B., Sweden).

Specific binding of iodinated ovine growth hormone (oGH) and ovine placental lactogen (oPL) to hepatic microsomal membranes was performed as described previously (Breier *et al.* 1994*b*). Data are presented as specific binding per mg protein of the membrane preparation.

5.3.5 Statistical analyses

Data arising from single-time measurements on the animals (carcass weight, measurements and dimensions of carcass and hind leg, organ weights) were subjected to two-way analysis of variance to test effects of treatment, treatment period and their interaction. Where appropriate, carcass weight was used as a covariate. The same statistical model was also applied to data from IGF-II, haematological and nitrogen balance studies with the pretreatment values of corresponding parameters as covariates (for IGF-II and haematological data only). Hepatic binding data were subjected to one way of analysis since only one age group was involved. Data arising from repeated measurements on animals (liveweight and metabolite/hormone concentrations) were subjected to multivariate (repeated measures) analysis of variance to test the effects of treatment, time (repeated factor) and their interaction. Metabolite/hormone concentrations measured on more than one occasion within a day were pooled because there were no significant interactions between treatment and time (within a day).

Pre- and post-challenge concentrations of hormones and metabolites were subjected to multivariate analysis of variance to test the effects of treatment (saline vs IGF-I), treatment period (day 49 vs day 77 of IGF-I treatment), block (A and B) and their interactions with sampling time. Preliminary analysis showed that there were no effects of treatment period and block or their interactions with sampling time. Therefore, results were pooled across both treatment period and block within treatment. Data during the post-challenge periods were then subjected to multivariate (repeated measures) analysis of variance to test the effects of treatment, time (repeated factor) and their interaction. Areas under the curve (AUC) during the post-challenge period were calculated by triangulation. Post-challenge glucose curves in the glucose and insulin challenges were further analysed to determine the fractional decay constant (K) by a non-linear, least squares Gauss-Newton iterative method (i.e fitting $G(t)=G(0)e^{-Kt}$ where G(t) is the concentration of glucose at time t, G(0) is the concentration at t=0). Over 99% of the

variation in glucose concentration with time in each sheep was accounted for by this model. Statistical analyses were conducted using the computer packages 'SAS' (1986) and 'REG' (Gilmour, 1985). All data are presented as least square means and standard errors (covariate-adjusted where appropriate).

One animal in the 8-week saline treatment group died early in the trial and was not replaced.

5.4 RESULTS

5.4.1 Plasma concentrations of hormones and metabolites

Circulating concentrations of IGF-I (Figure 5.1) remained low and stable in the control group throughout the study. The mean basal IGF-I concentration was 115.4±3.7 ng/ml compared to 181.4±8.4 ng/ml in our previous study with well-fed lambs of a similar age (Cottam *et al.* 1992). In sheep treated with IGF-I, levels rose significantly ($P<0.001$) from 119.4±5.1 ng/ml at day 0 to 264.6±8.3 ng/ml at day 14 of treatment and continued to increase as the treatment proceeded. As a result, there was a significant ($P<0.001$) interaction between treatment and time. Plasma concentrations of IGF-II were lower ($P<0.05$) in IGF-I treated than in control animals at both week 8 (457.2±32.2 ng/ml vs 541.6± 35.1 ng/ml) and week 12 (406.7± 31.7 ng/ml vs 523.5±31.5 ng/ml) of treatment. Plasma concentrations of GH (Figure 5.1) were significantly ($P<0.05$) depressed by IGF-I treatment. Plasma insulin concentrations were below the assay limit (50 pg/ml) in both groups throughout this study (data not shown).

Treatment with IGF-I had no effects on circulating concentrations of glucose (Figure 5.2) but did significantly ($P<0.01$) reduce plasma concentrations of creatinine and tended to reduce plasma concentrations of urea (Figure 5.2).

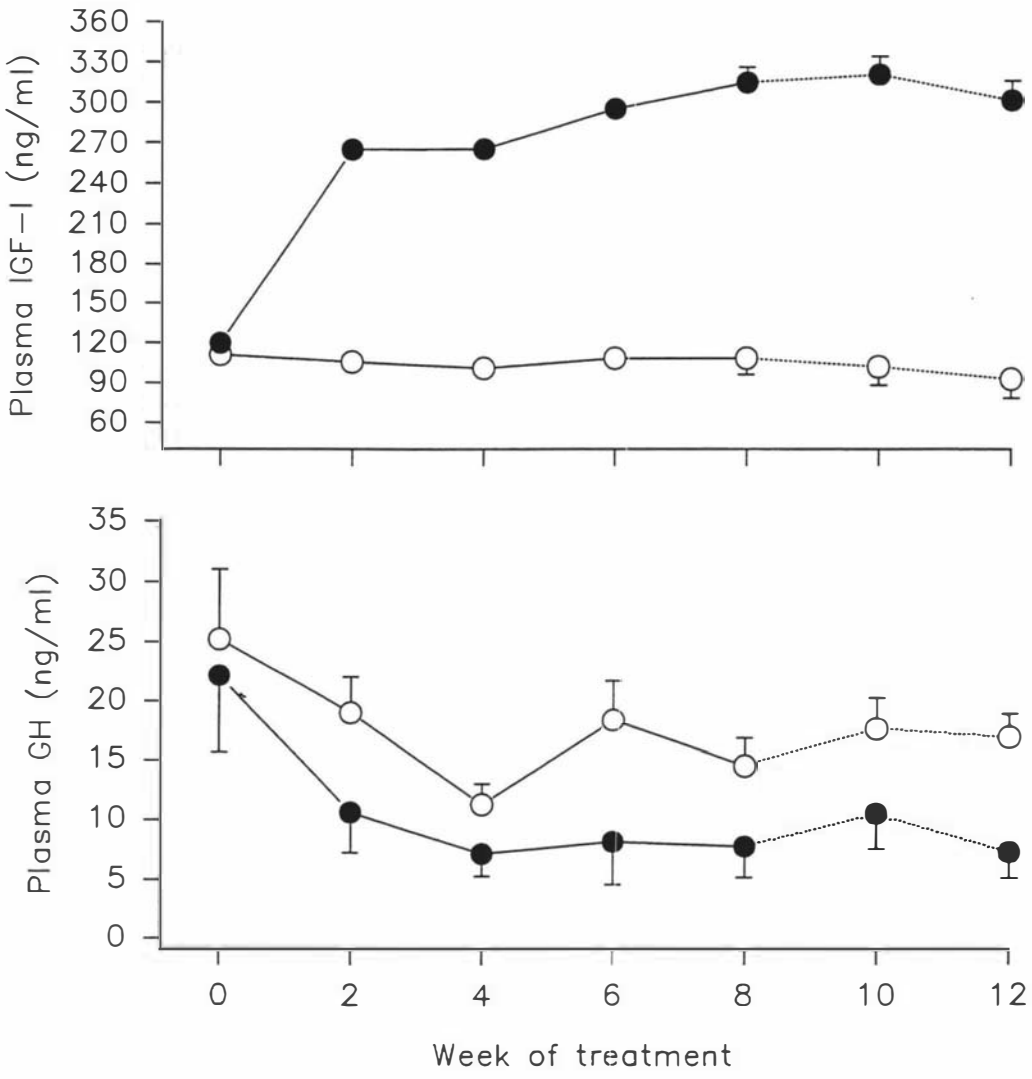

Figure 5.1 Effects of saline (○) and IGF-I (●) treatment on plasma concentrations of IGF-I and growth hormone in energy restricted sheep. Vertical bars represent the standard error of the mean. Means for each point from week 2 to week 12 are covariate-adjusted to a common pretreatment mean (week 0). The means of 15 or 16 observations are represented by solid lines and the means of 8 observations represented by broken lines.

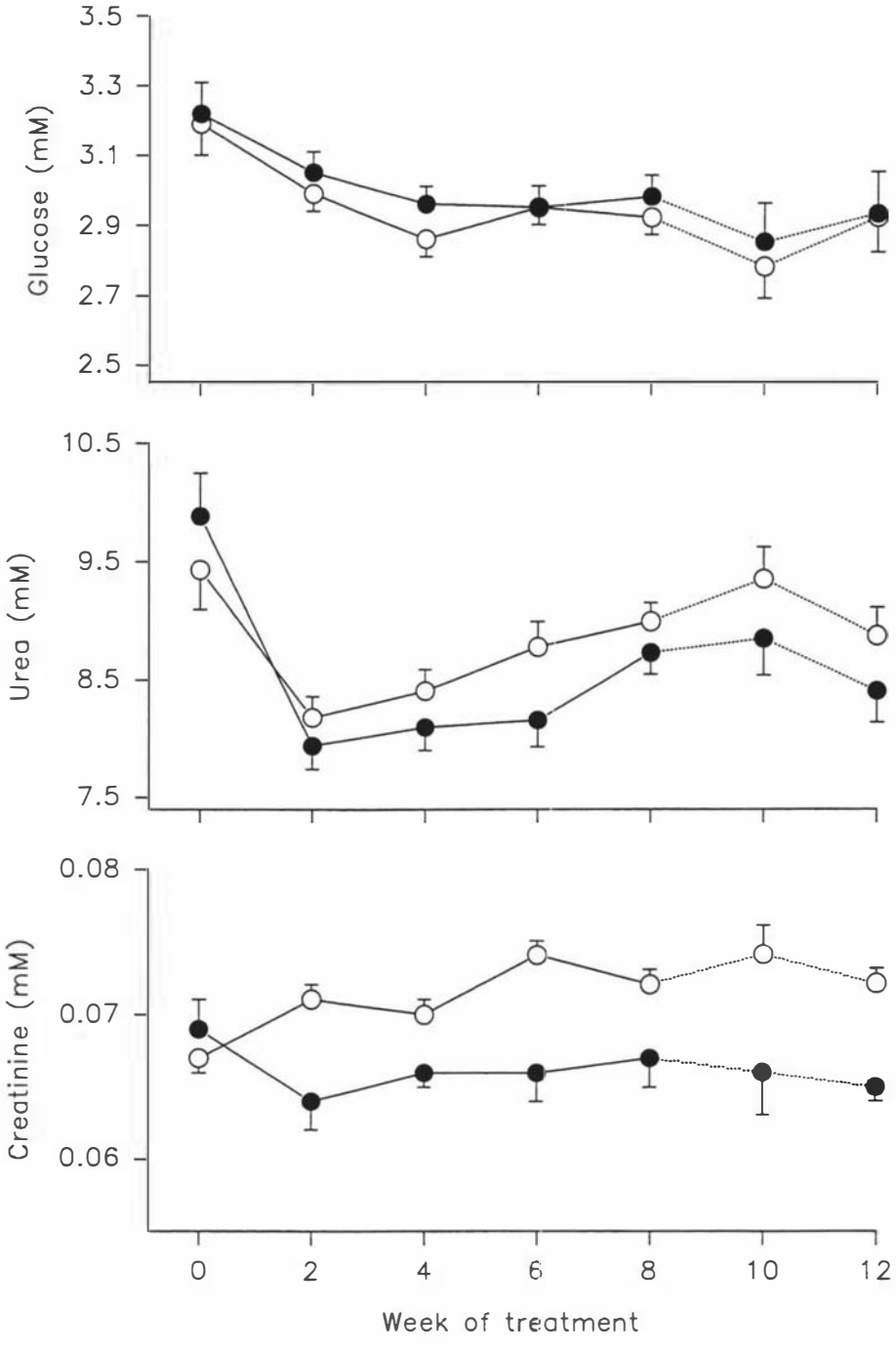

Figure 5.2 Effects of saline (○) and IGF-I (●) treatment on plasma concentrations of glucose, urea and creatinine in energy restricted sheep. Vertical bars represent the standard error of the mean. Means for each point from week 2 to week 12 are covariate-adjusted to a common pretreatment mean (week 0). The means of 15 or 16 observations are represented by solid lines and the means of 8 observations represented by broken lines.

5.4.2 Metabolic challenges

Baseline glucose concentrations prior to the glucose challenge were similar between the two treatment groups (data not shown). Glucose concentrations peaked at 5 min post-challenge and approached baseline levels by 60 min post challenge. The fractional decay constant (K) for glucose clearance was not affected by IGF-I treatment (Table 5.2).

Table 5.2. Effects of saline (control) and IGF-I on glucose fractional decay constant (K), and areas under the curve (AUC) for glucose, NEFA, insulin and GH following metabolic challenges (mean±SE)

Parameters	Control	IGF-I
n	15	16
Glucose challenge		
K (min^{-1})	0.016±0.001	0.017±0.001
$AUC_{insulin}$ (min.pg/ml)	237.3±29.5	237.8±28.6
Insulin challenge		
K (min^{-1})	0.0234±0.0008	0.0242±0.0008
Epinephrine challenge		
$AUC_{glucose}$ (min.mM)	3.93±0.12	3.97±0.12
AUC_{NEFA} (min.ueq/l)	0.249±0.028[a]	0.345±0.027[b]
GRF challenge		
AUC_{GH} (min.ng/ml)	31.6±3.6	21.6±3.7

[ab] Means with different superscripts are significantly different ($P<0.05$)
AUC = area under the response curve, calculated by triangulation

Insulin concentrations were also stable prior to the glucose challenge and were not influenced by IGF-I treatment. Following the challenge, insulin concentrations rose

rapidly to a peak at 5 to 10 min and then returned to baseline levels by 60 min. Plasma concentrations of insulin during the challenge were not significantly affected by IGF-I treatment although peak concentrations tended to be higher in the IGF-I group. Similarly, AUC for insulin was not affected by IGF-I treatment (Table 5.2).

Glucose concentrations prior to the insulin challenge were significantly higher in the IGF-I treated than in saline-treated animals (Figure 5.3). Following the challenge, glucose concentrations declined rapidly to a minimum at 40 min post-challenge. There were no significant effects of IGF-I treatment on plasma glucose responses to an insulin load as measured by the fractional decay constant for glucose (Table 5.2).

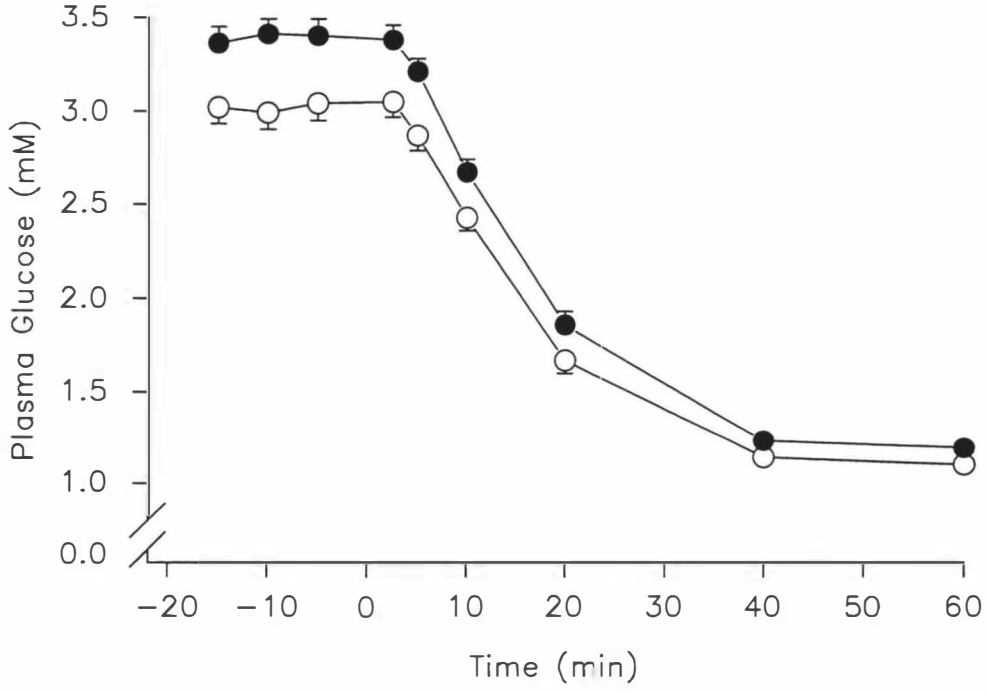

Figure 5.3 Effects of saline (○) and IGF-I (●) treatment on plasma concentrations of glucose during the insulin challenge. Vertical bars represent the standard error of the mean.

Circulating concentrations of glucose prior to the epinephrine challenge were marginally ($P<0.10$) greater in IGF-I treated than in saline-treated animals (Figure 5.4). However, the difference between the groups disappeared during the post-challenge period. AUC for glucose was similar for the two groups (Table 5.2).

Plasma NEFA concentrations prior to the epinephrine challenge were not different between the two groups (Figure 5.4) but were significantly ($P<0.05$) greater in the IGF-I

group than in the saline group over the interval 20 to 60 minutes post-challenge. Such a difference led to a 39 % greater AUC ($P<0.05$) for NEFA in the IGF-I group (Table 5.2).

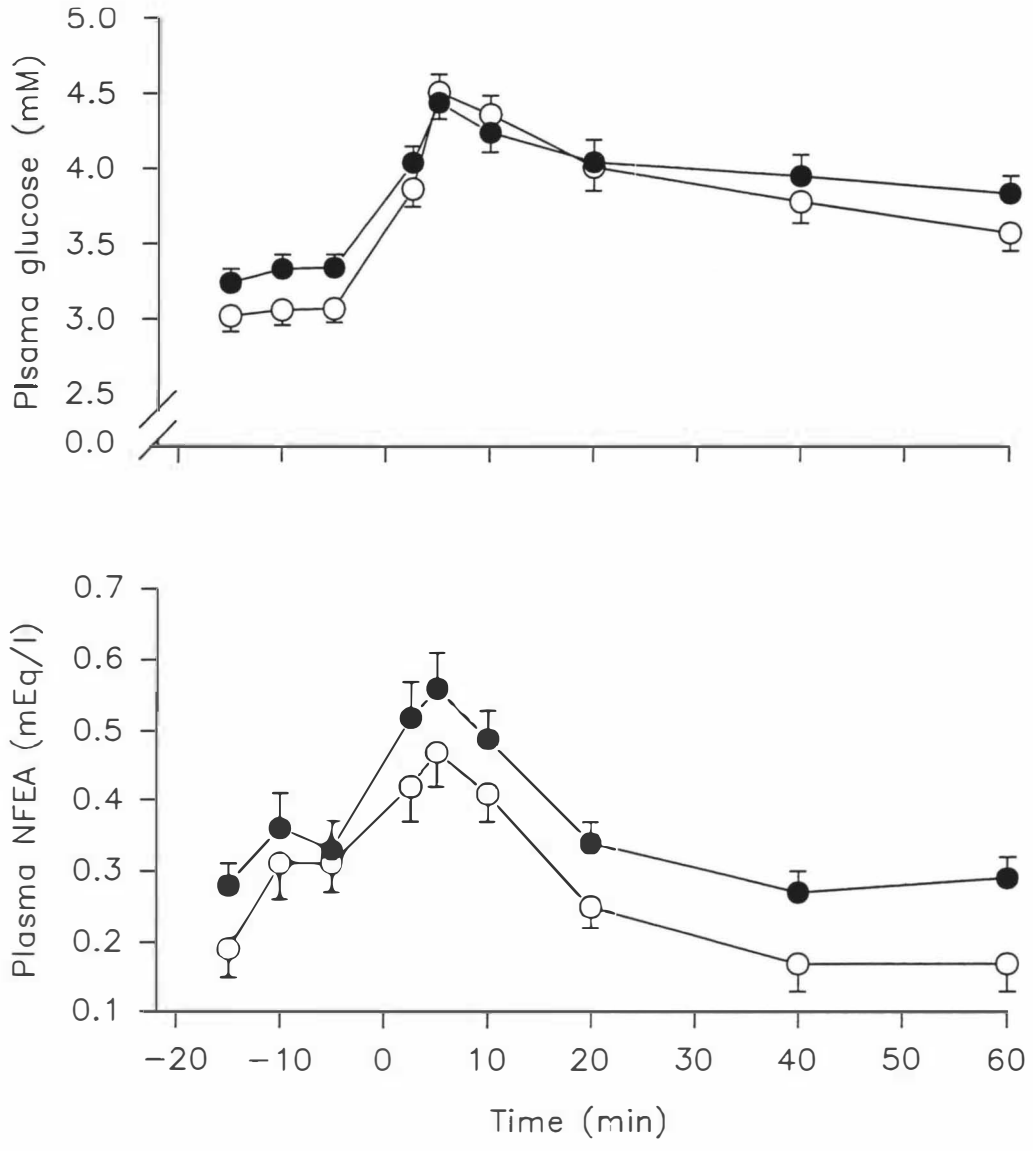

Figure 5.4 Effects of saline (○) and IGF-I (●) treatment on plasma concentrations of glucose and NEFA during the epinephrine challenge. Vertical bars represent the standard error of the mean.

Circulating concentrations of GH prior to the GRF challenge were similar between the IGF-I and saline groups (Figure 5.5). Following challenge, there was a biphasic increase in plasma GH concentrations. The first minor peak occurred within 5-10 min, and was followed by a second major peak 20 min after challenge. Thereafter, circulating

concentrations of GH declined slowly to approach baseline levels at 60 min post-challenge. AUC for GH tended to be lower (P<0.1) in the IGF-I group than in the control group (Table 5.2).

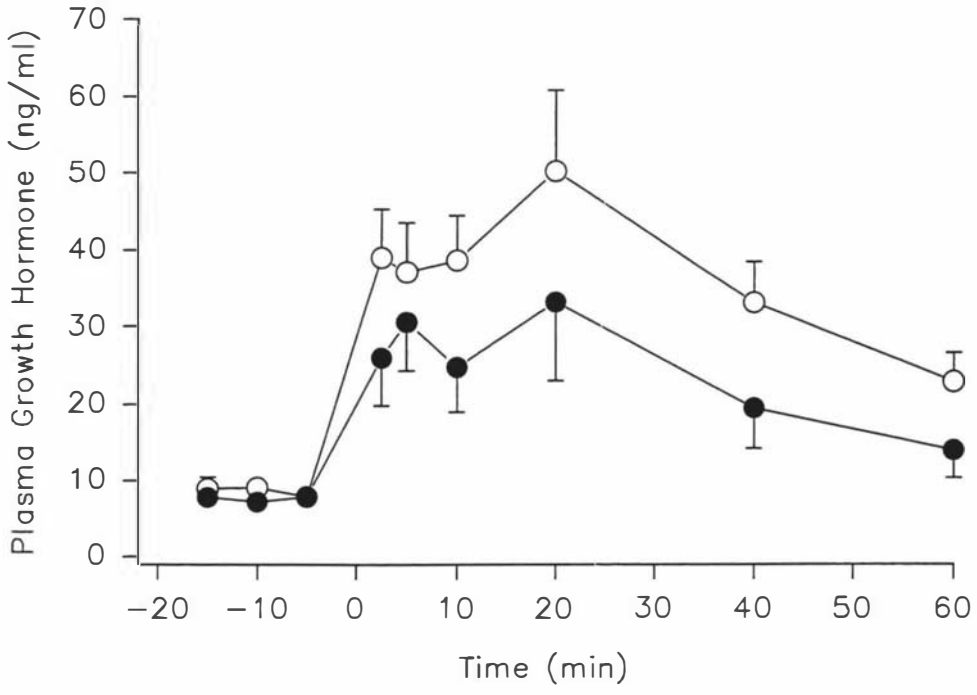

Figure 5.5 Effects of saline (○) and IGF-I (●) treatment on plasma concentrations of GH during the GRF challenge. Vertical bars represent the standard error of the mean.

5.4.3 Hepatic binding of oPL and oGH

Hepatic binding was measured only for the 8-week group. Treatment with IGF-I reduced the specific binding of ^{125}I-labelled oPL (P<0.001) and oGH (P<0.01) to hepatic membrane preparations relative to those of the control group (Table 5.3).

Table 5.3 Effects of saline (control) and IGF-I administered for 8 weeks on specific binding (SB) of ^{125}I-labelled oGH and ^{125}I-labelled oPL to hepatic membrane preparations (mean±SE)

Parameter	Control	IGF-I
n	7	8
^{125}I-oGH binding (%SB/mg protein)	1.02±0.05^{a}	0.74±0.05^{b}
^{125}I-oPL binding (%SB/mg protein)	2.39±0.10^{c}	1.75±0.10^{d}

^{ab} Means with different superscripts are significantly different (P<0.01)

^{cd} Means with different superscripts are significantly different (P<0.001)

5.4.4 Haematological profiles

Haematological parameters are summarised in Table 5.4. Relative to those of saline-treated sheep, IGF-I treatment significantly (P<0.05) increased haemoglobin and PCV, while having no significant effects on mean corpuscular haemoglobin concentrations (MCHC). Leucocyte concentrations and counts of neutrophils and lymphocytes were not affected by treatment with IGF-I. However, treatment significantly (P<0.05) increased counts of eosinophils (0.33±0.04 x10^{9}/l) relative to those of saline-treated sheep (0.18±0.05 x10^{9}/l). There were no significant effects of treatment period on any of these parameters.

5.4.5 Nitrogen balance

The average daily nitrogen intakes of the control and IGF-I groups were not different (Table 5.5) but the excretion of nitrogen via faeces was significantly (P<0.05) lower in the IGF-I treated animals (7.58±0.13 g/day) than in the control animals (7.99±0.14 g/day). As a result, IGF-I treatment significantly increased nitrogen digestibility relative to that of controls (control, 73.0±0.04%; IGF-I-treated, 74.4±0.04%, P<0.05). Nitrogen excretion via urine was not significantly affected by IGF-I treatment although it tended to be lower in IGF-I treated sheep. The net result was a small (17 %), but non-significant, increase in N balance (retention) in the IGF-I treated group.

Table 5.4 Effects of saline (control) and IGF-I administered for 8 or 12 weeks on haematological parameters at the end of treatment in energy restricted sheep (mean±SE)

Parameter	8-week[d]		12-week[d]	
	Control	IGF-I	Control	IGF-I
n	7	8	8	8
Leucocytes (x10^9/l)	7.70±0.49	7.62±0.45	6.88±0.46	8.10±0.46
Haemoglobin (g/dl)	10.86±0.28[b]	11.43±0.26[c]	9.83±0.26[a]	10.62±0.26[b]
PCV (l/l)	0.317±0.007[a]	0.332±0.007[b]	0.318±0.007[a]	0.340±0.007[b]
MCHC (g/dl)	34.3±0.3[a]	34.2±0.2[a]	30.9±0.2[b]	31.2±0.2[b]
Leucocyte differential counts (x10^9/l)				
Neutrophils	1.40±0.26	1.61±0.24	0.97±0.20	1.42±0.24
Lymphocytes	5.98±0.52	5.56±0.49	5.79±0.49	6.38±0.49
Eosinophils	0.24±0.07[a]	0.35±0.06[b]	0.12±0.06[a]	0.31±0.06[b]

[abc] Means with different superscripts are significantly different (P<0.05)

[d] Value at day 56 (8-week) or 84 (12-week) of treatment adjusted to common pre-treatment values

Table 5.5 Effects of saline (control) and IGF-I administered for 8 or 12 weeks on nitrogen balance in energy restricted sheep (means±SE)

Parameter	8-week		12-week	
	Control	IGF-I	Control	IGF-I
n	7	8	8	8
N intake (g/day)[c]	29.4±0.02[a]	29.4±0.02[a]	29.9±0.02[b]	29.9±0.02[b]
Faecal N (g/day)	7.82±0.20[a]	7.14±0.19[b]	8.15±0.19[a]	8.03±0.19[a]
N digestibility (%)	0.730±0.006[a]	0.750±0.006[b]	0.730±0.006[a]	0.740±0.006[ab]
Urinary N (g/day)	15.9±0.60[a]	15.0±0.56[a]	17.1±0.56[b]	16.6±0.56[b]
N balance (g/day)	5.23±0.61	6.44±0.58	5.25±0.58	5.83±0.58

[ab] Means with different superscripts are significantly different ($P<0.05$)

[c] Adjusted to a common intake immediately prior to the commencement of nitrogen balance study

5.4.6 Liveweight and organ weights

Changes in liveweight during treatment are depicted in Figure 5.6. Initial liveweight tended to be lower in the IGF-I group than in the control group (41.9±0.6 vs 43.2±0.6 kg) although such differences were not significant ($P>0.10$). During week 2 to 8 of treatment liveweights of saline-treated sheep remained unchanged, while those of IGF-I treated sheep tended to increase. This resulted in a significant ($P<0.05$) difference in liveweight between the two groups at week 8 of treatment (saline group, 43.4±0.2 kg; IGF-I group, 44.0±0.2 kg). However, these differences, although maintained between the two groups, were not significant in the 12-week animals alone (there being only 8 animals per group at that stage). The growth rates of up to 50 g/day compare with those of about 250 g/day in animals of similar liveweight consuming twice maintenance in our previous study (Cottam *et al.* 1992).

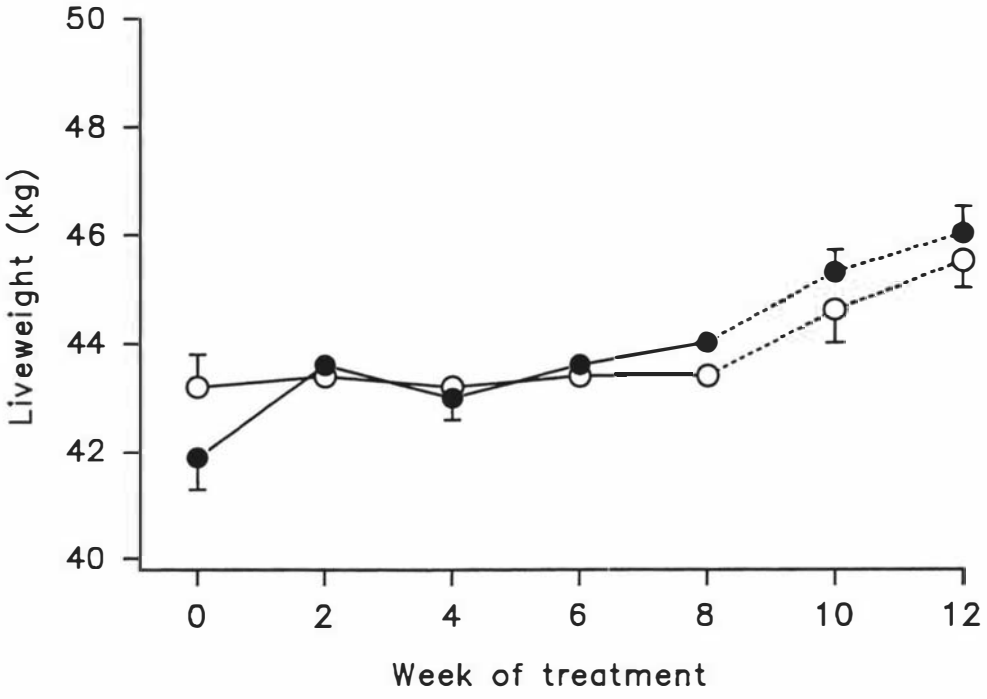

Figure 5.6 Effects of saline (○) and IGF-I (●) treatment on liveweight in energy restricted sheep. Vertical bars represent the standard error of the mean. Means for each point from week 2 to week 12 are covariate-adjusted to a common pretreatment mean (week 0). The means of 15 or 16 observations are represented by solid lines and the means of 8 observations represented by broken lines.

Weights of organs are shown in Table 5.6. Relative to those of saline-treated sheep, IGF-I treatment for both 8 and 12 weeks significantly ($P<0.001$) increased mean weight of the kidneys, spleen (particularly at 12 weeks), thymus and mandibular salivary gland.

Table 5.6 Effects of saline (control) and IGF-I administered for 8 or 12 weeks on organ weights in energy restricted sheep (mean±SE)

Organ weight (g)	8-week		12-week	
	Control	IGF-I	Control	IGF-I
n	7	8	8	8
Liver[d]	584.7±14.3	555.5±13.3	572.4±13.9	557.3±13.1
Kidneys[d]	101.9±3.1[a]	114.5±2.8[b]	104.6±3.0[a]	113.9±2.8[b]
Heart[d]	203.3±8.9	217.2±8.7	213.7±8.7	224.6±8.2
Lungs	424.9±20.3	429.0±18.8	412.9±19.8	481.5±18.6
Spleen[d]	57.5±3.8[a]	68.6±3.5[b]	59.6±3.7[a]	81.9±3.5[c]
Pancreas	55.3±3.3	57.0±3.1	55.4±3.1	60.7±3.1
Thymus[d]	13.5±1.6[a]	18.1±1.5[b]	14.6±1.6[a]	21.1±1.6[c]
Thyroid	4.98±0.48	5.80±0.45	5.44±0.47	5.99±0.44
Adrenal	3.43±0.22	3.55±0.20	3.79±0.20	3.75±0.20
Salivary, parotid	9.28±0.48	10.1±0.45	9.78±0.45	10.1±0.45
Salivary, mandibular[d]	14.0±0.9[a]	18.8±0.8[b]	16.1±0.8[a]	18.6±0.8[b]

[abc] Means with different superscripts are significantly different (P<0.01)

[d] Adjusted to a common carcass weight

5.4.7 Carcass parameters and hind leg composition

Administration of IGF-I for 8 or 12 weeks had no significant effects on carcass parameters (Table 5.7). However, IGF-I treatment decreased weights of dissectible fat in the hind leg at week 8 and caused a further reduction at week 12 (Table 5.8). The overall effects of IGF-I treatment on weights of dissectible fat were significant (control, 477.4±13.0 g; IGF-I treated, 417.4±12.6 g, P<0.01). Treatment with IGF-I also significantly (P<0.05) reduced weights of tibia compared to those of control sheep.

Otherwise, there were no significant effects of treatment or treatment period on hind leg parameters.

Table 5.7 Effects of saline (control) and IGF-I administered for 8 or 12 weeks on carcass parameters in energy restricted sheep (mean±SE)

Parameter (unit)	8-week		12-week	
	Control	IGF-I	Control	IGF-I
n	7	8	8	8
Carcass weight (kg)	19.5±0.3	20.3±0.3	20.1±0.3	19.7±0.3
Body length (mm)[c]	1017.4±8.0[a]	1007.7±7.4[a]	1047.0±7.9[b]	1062.0±7.4[b]
Shoulder width (mm)[c]	177.5±2.5	175.8±2.3	172.2±2.5	174.6±2.3
Gigot width (mm)[c]	238.7±3.8	239.7±3.2	244.3±3.4	246.7±3.2

[ab] Means with different superscripts are significantly different (P<0.001)

[c] Adjusted to a common carcass weight

Table 5.8 Effects of saline (control) and IGF-I administered for 8 or 12 weeks on hind leg components in energy restricted sheep (mean±SE)

Parameter	8-week		12-week	
	Control	IGF-I	Control	IGF-I
n	7	8	8	8
Hind leg (g)				
Weight[d]	3026.4±83.6	3072.1±78.2	3177.1±78.2	2967.9±78.2
Dissectible muscle[d]	2051.9±38.1	2108.3±35.4	2028.3±37.7	2064.1±35.4
Dissectible bone[d]	497.6±14.3	497.3±13.3	499.7±14.2	510.6±13.3
Dissectible fat[d]	485.1±19.0[a]	447.9±17.6[b]	469.7±18.8[a]	386.8±17.7[c]
Bones				
Femur length (mm)	172.2±2.6	172.4±2.4	173.8±2.4	176.7±2.4
Tibia length (mm)	188.8±2.9	192.5±2.7	195.8±2.7	194.8±2.7
Femur wt (g)[e]	151.3±3.4	147.3±3.1	151.4±3.1	149.4±3.2
Tibia wt (g)[e]	121.3±3.2[a]	116.5±3.0[b]	123.9±3.0[a]	116.5±3.0[b]

[abc] Means with different superscripts are significantly different ($P<0.05$)

[d] Adjusted to a common carcass weight

[e] Adjusted to a common bone length

5.5 DISCUSSION

It was shown previously that chronic administration of IGF-I has virtually no growth-promoting effects in well fed normal growing sheep (Cottam *et al.* 1992). This finding is inconsistent with the well known anabolic role of IGF-I in GH-deficient animals and suggests that IGF-I treatment may have little effect in conditions where circulating concentrations of IGF-I are already high. Consistent with this, Schalch *et al.*

(1989) reported that exogenous IGF-I treatment had growth-promoting effects in energy-restricted, but not in *ad libitum* fed, rats. The aim of this study was, therefore, to evaluate the impact of chronically administered IGF-I on body growth, composition and endocrine and metabolic responses of sheep under near-maintenance conditions in which circulating concentrations of IGF-I were expected to be low. Under catabolic conditions when circulating IGF-I levels are low, short-term infusion of IGF-I has been shown to have anabolic effects in several species including sheep (Douglas *et al.* 1991; Koea *et al.* 1992).

In the present study, the sheep were fed at or near maintenance. Under these conditions, increased rates of growth can be achieved only if the feed is digested more efficiently or absorbed nutrients are partitioned in a different manner such that the composition of growth is altered to favour tissues with a low energy density per unit weight (eg muscle) over those with a high energy density (eg fat).

In this study, IGF-I treatment significantly improved digestibility of nitrogen. However, the effect was small and was associated with only a small (and non-significant) increase in nitrogen balance in IGF-I treated animals. A recent study showed that a 14 day systemic infusion of recombinant IGF-I led to significant increases in the size of the villi, crypts and *muscularis* lining of the jejunum of normal female rats (Conlon *et al.* 1995), suggesting that IGF-I may improve feed conversion efficiency by increasing absorptive capacity of the gut. Given the fact that changes in nitrogen balance were small in our IGF-I treated animals, it is not surprising that there was little effect of IGF-I treatment on body growth rate. Although IGF-I treatment caused a decrease in concentrations of creatinine and (to a lesser extent) urea, which could imply reduced turnover of muscle and improved efficiency of nitrogen deposition in muscle, such effects may be due simply to changes in renal plasma flow and glomerular filtration rate, as reported previously (Guler *et al.* 1989*b*).

There was, however, a significant decrease in fat content of the hind leg with IGF-I treatment. Consistent with this, the NEFA response to an epinephrine load was significantly greater in the IGF-I group than in the saline group, suggesting that IGF-I treatment increased the sensitivity of adipose tissue to a lipolytic stimulus. A similar finding has been reported in other species such as chickens (Tixier-Boichard *et al.* 1992), rats (Tomas *et al.* 1993) and minipoodles (Guler *et al.* 1989*a*). It has been

suggested that effects of IGF-I on fat repartitioning are indirectly mediated via suppression of circulating insulin concentrations (Tixier-Boichard *et al.* 1992; Hussain *et al.* 1994). In the present study, however, circulating concentrations of insulin were below the assay limit which leaves little opportunity for their further suppression by IGF-I treatment and suggest that other mechanisms were involved. Further, IGF-I did not affect the insulin response to a glucose load.

While IGF-I has been shown to be growth-promoting in malnourished rats (Schalch *et al.* 1989; Yang *et al.* 1990), those studies used supraphysiological doses (10 x) relative to those used here. It is likely that the ability of animals in the present study to repartition absorbed nutrients in favour of muscle at the expense of fat may have been limited by the negative effects of IGF-I treatment on circulating concentrations of GH. Such negative effects of IGF-I on plasma GH concentrations seem to be mediated via suppressed GH secretion since the release of GH in response to the GRF challenge was lower in the IGF-I group than in the control group. Depressed GH concentrations with IGF-I treatment have been reported previously in rats (Sato & Frohman, 1993), chickens (McGuinness & Cogburn, 1991) and humans (Hartman *et al.* 1993). Treatment with IGF-I in the present study also down-regulated hepatic GH receptors. This effect, which has not been reported previously, would have further reduced the ability of GH to repartition nutrients in favour of growth if evident in other tissues such as adipose tissue. Undernutrition is commonly associated with high circulating concentrations of GH which favour the retention of protein at the expense of fat (Beermann *et al.* 1990). In this case, however, high circulating concentrations of IGF-I were associated with reduced concentrations of GH, a situation which may have limited fat mobilization and hence reduced the ability of these animals to oxidise fat and spare amino acids for protein synthesis.

The observation that chronic IGF-I administration down-regulates the hepatic GH receptor is novel. The possibility of a short feedback loop of endocrine IGF-I on the hepatic GH receptor (GHR) has not been investigated previously. In GH-deficient dwarf rats, hepatic growth hormone receptor mRNA levels are elevated and are reduced by either GH or IGF-I treatment (B. H. Breier, A. A. Butler, P. D. Gluckman, personal communication), an observation which is compatible with the present study. Thus, the feedback by IGF-I may include the direct inhibition of GHR synthesis in liver in

addition to the inhibition of pituitary GH secretion. It is not clear whether the effects of IGF-I on the GHR are direct or mediated by suppression of insulin. The latter may be less likely since peripheral insulin levels were below the level of detectability in both groups: however levels in the portal circulation may have differed.

In the previous study from this laboratory (Cottam *et al.* 1992), persistent mild hyperglycaemia was observed in well fed sheep treated with IGF-I. It was suggested that this was a consequence of suppression of insulin secretion at doses of IGF-I which were sufficiently low for the insulin-like effects of IGF-I to be noted. Hyperglycaemia was not observed in the present study presumably because insulin levels were already suppressed by the undernutrition. It has previously been shown that direct effects of IGF-I on glucose clearance depend on elevated circulating free IGF-I levels (Douglas *et al.* 1991). The lack of a consistent effect of IGF-I treatment on either basal glucose concentrations or on the disappearance rate of glucose after glucose challenge suggests that the effects of IGF-I observed in the present study were not due to insulin-like action associated with elevated free IGF-I levels. A further possible explanation of the failure of IGF-I to elicit a growth response in the present study is that both insulin and free IGF-I levels in plasma were low. Higher levels leading to a higher free IGF-I concentration or co-administration of insulin or GH to induce a hyperinsulinaemic response might have allowed a growth response.

Some of the most marked effects associated with IGF-I treatment are selective organomegaly, particularly with regard to spleen and thymus. These were again observed in the present study. It is of interest that the effect of IGF-I on the salivary gland is restricted to the submandibular gland. It is not known whether the increase in weight is due to an increase in lymphoid activity within the gland or to effects on the exocrine component. Treatment with IGF-I did not alter total leucocyte counts but did increase eosinophils. Since eosinophils are implicated as effector cells in the control of parasites, these data suggest that IGF-I treatment might increase resistance to parasite infection. Consistent with this, Presson *et al.* (1988) observed that sheep resistant to the intestinal parasite *Haemonchus contortus* had increased thymus weights and greater numbers of globule leucocytes in abomasal tissue than susceptible sheep. In another study with rats (Clark *et al.* 1993), where the dose of IGF-I used was 25 times greater than that used in the present study, IGF-I treatment caused substantial responses in several immune

parameters, including leucocyte numbers.

In conclusion, these data show that IGF-I treatment does not have marked effects on growth rate or body composition in sheep fed at or near maintenance, extending previous findings in well-fed sheep (Cottam *et al.* 1992). The lack of a growth-promoting effect of IGF-I in the present study may be explained in part by negative feedback effects on both circulating GH concentrations and hepatic GH receptor numbers. This suggests that chronic IGF-I usage in endocrinologically normal animals or subjects, at least at low dosage, will not lead to enhanced growth or anabolism.

CHAPTER 6

ONTOGENY OF IGF-I RESPONSIVENESS TO bGH IN YOUNG LAMBS

The responsiveness of young lambs to exogenous GH, as measured by plasma IGF-I concentrations, was found not to vary greatly over the first 9 weeks of life but was significantly lower than in yearlings. This was reflected by a corresponding response of hepatic GHR to GH stimulus over the same period.

6.1 ABSTRACT

It was previously shown that GH treatment had only a small effect on circulating concentrations of IGF-I in very young lambs (Chapter 4), indicating that hepatic GHR may be not fully functional. Therefore, the ontogeny of hepatic GHR, as measured by responses of both plasma IGF-I and hepatic GHR to an exogenous GH stimulus, was examined using sheep of different ages (days 1-7, 14-21, 28-35 and 56-63 of life, and yearlings). The IGF-I response to bGH was first examined in yearling sheep using two doses of bGH (0.1 and 0.2 mg/kg LW/day). Based on these results, lambs in four groups up to day 63 of life were treated by twice daily subcutaneous injection for 5 days with bGH (n=10) at a dose of 0.15 mg/kg LW/day or saline (n=10). Jugular blood samples were taken once daily on days -1, 4 and 5 of treatment and analysed for plasma concentrations of GH, IGF-I, insulin, glucose, NEFA and urea. Plasma concentrations of insulin, glucose and urea were high at day 6/7 of life and decreased with increasing age, whereas no such effects were apparent in the other parameters. In general, all plasma parameters in yearling sheep were low relative to those in lambs except plasma insulin and urea concentrations, both of which were similar to those in lambs. bGH treatment in lambs up to day 63 of life had little effect on plasma concentrations of GH, insulin, glucose or urea, but significantly ($P<0.05$) increased circulating concentrations of IGF-I at all ages and of NEFA at day 62/63 of life. In contrast, GH treatment at either dose in yearlings significantly increased these parameters except plasma urea concentrations which were decreased in bGH-treated yearlings. However, the responses of plasma IGF-I concentration to GH stimulus in lambs up to day 63 of life were small compared to those in yearling sheep. Consistent with this, GH treatment failed to affect hepatic GH binding in young lambs, but up-regulated it in yearling sheep. Furthermore, basal (unstimulated) GH binding did not differ between sheep of 7 vs 63 vs 365 days of age, despite the greater IGF-I responses to GH in the latter group. It is concluded that hepatic GHR in lambs up to day 63 of life are not fully functional compared to the situation in yearlings.

6.2 INTRODUCTION

Although young lambs are reported to exhibit adult-like binding of GH to hepatic receptors (Gluckman *et al.* 1983), it was shown that lambs less than three weeks old do

not respond to exogenous GH with substantial increases in circulating insulin-like growth factor-I (IGF-I) concentrations (Chapter 4). The same preparation of GH did, however, significantly increase IGF-I levels in pregnant ewes (Chapter 2). Thus, young lambs show a very blunted IGF-I response to GH compared with older sheep. This suggests that, in young lambs, the hepatic GH receptors (GHR) are present but non-functional (or have limited functionality). The consequent inability of young lambs to secrete increased levels of IGF-I in response to an exogenous GH stimulus may account for the lack of growth-promoting effects of GH in animals of this age (Sun *et al.* 1991; Chapter 4) although it does not explain how GH stimulates wool growth (Sun *et al.* 1991) or oPL stimulates body growth (Chapter 4).

Levels of IGF-I in plasma following GH stimulation depend on the interaction of GH and the hepatic receptor, and subsequent secretion of IGF-I by the liver. The objective of this study was, therefore, to investigate the ontogeny of hepatic GHR in young lambs by measuring IGF-I responsiveness to GH as well as direct measurement of hepatic GH binding.

6.3 MATERIALS AND METHODS

6.3.1 Animals and treatment

Twenty Border Leicester x Romney lambs (mixed sex) were used at each of four ages (days 1-7, 14-21, 28-35, and 56-63 of life) and a further 21 animals were used at one year of age. Each group was exposed to a two-day pretreatment and five-day treatment period. Lambs up to day 63 of life remained with their dams on pasture throughout the experiment. The yearling animals were housed individually and fed a diet of sheep nuts and lucerne chaff *ad libitum.* Fresh water was available *ad libitum.* Animals were weighed at the beginning (day -1 of treatment) and end (day 5 of treatment) of the experiment.

The yearlings, all of which were castrated males, were randomly assigned to three equal experimental groups and were treated first, with saline (n=7), or with bGH at 0.1 mg/kg LW/day (n=7) or 0.20 mg/kg LW/day (n=7) to ensure that treatment was effective at increasing plasma IGF-I concentrations to expected values for mature animals. Based on these results, a dose of 0.15 mg/kg LW was chosen for the young

lambs. Animals of each age group up to day 63 of life were randomly assigned to two equal experimental groups balanced for sex and rearing rank. After a two-day pretreatment period, one group was treated with recombinantly-derived bGH (0.15 mg/kg LW/day) and the other with sterile physiological saline (0.15 ml/kg LW/day) for five days. Injections were made twice daily (0800h and 1600h) subcutaneously in the neck (on alternate sides), commencing with the first injection at 1600h on day 1. The bGH was dissolved in sterile carbonate buffered saline (pH 9.4) at a concentration of 2 mg/ml. Injection doses were determined based on liveweight at day -1 of treatment.

6.3.2 Blood sampling

Animals were blood sampled (1530h) by jugular venipuncture on days -1, 4 and 5 of treatment. Samples (10 ml) were withdrawn into vacutainers (Nipro Medical Industries, Japan) containing EDTA as the anticoagulant and immediately placed on crushed ice. Within 20 min of collection the samples were centrifuged at 3000 g and 4°C for 20 min. Plasma was harvested into duplicate vials and stored at -20°C for later analysis.

6.3.3 Chemical and hormonal analyses

Plasma concentrations of urea, glucose, NEFA, GH, IGF-I, and insulin were measured using methods described previously (Chapter 2).

6.3.4 Organ collection and measurements

Slaughter of the lambs commenced at 1300h (7 h after the last injection) on day 5 of treatment. Lambs were slaughtered within 1 h of separation from their dams and yearlings were slaughtered straight off feed. The animals were weighed, stunned with a captive bolt pistol and exsanguinated. The skin, head, feet and viscera were removed. The liver was dissected out, blotted dry and its fresh weight recorded. Samples of liver were then rinsed in saline and immediately frozen on dry ice for receptor studies.

6.3.5 Hepatic GH receptor measurements

Hepatic microsomal membranes were prepared as described previously (Sauerwein *et al.* 1991). Specific binding of iodinated ovine growth hormone (oGH) to hepatic

microsomal membranes was performed as described by Breier *et al.* (1994*a*). Data are expressed as specific binding per mg protein of the membrane preparation.

6.3.6 Statistical analyses

Plasma concentrations of metabolites and hormones were analysed by two-way analysis of variance, with treatment and age being the main effects. Plasma parameters on day 4 and 5 of treatment were averaged and adjusted for pre-treatment values by covariance analysis. Data are expressed as least square means and standard errors for all parameters, with yearlings referred to by their nominal age of 365 days. The same statistical analysis was also applied to hepatic growth hormone receptors (but without a covariate). Statistical analyses were conducted using the computer package 'SAS' (1986).

6.4 RESULTS

6.4.1 Plasma concentrations of GH, IGF-I and insulin

Effects of age and GH treatment on plasma concentrations of GH at the end of treatment are presented in Table 6.1. Plasma concentrations of GH in the control (saline-treated) group were similar across ages up to 63 days of life although the GH concentrations in lambs were very high compared to those in yearling sheep. In young lambs, GH treatment had no significant effects on circulating concentrations of GH 7.5 h after injection, although it marginally increased plasma concentrations of GH in lambs at days 20/21 and 34/35 of life. By contrast, in yearling sheep, GH treatment at the low dose (0.1 mg/kg LW) increased plasma concentrations of GH six-fold, and a further increase was evident at the high dose (0.2 mg/kg LW).

Plasma concentrations of IGF-I in saline-treated groups were similar in lambs up to 63 days of life although, like plasma GH levels, they were much higher in lambs than in yearling sheep (Table 6.2). GH treatment significantly ($P<0.01$) stimulated circulating concentrations of IGF-I in young lambs, responses being similar across ages. Exogenous GH also increased circulating concentrations of IGF-I in yearling sheep. However, the magnitude of the IGF-I response was much greater in yearlings (a three-fold increase) than in lambs (an increase of 30-40%).

Table 6.1 Effects of age and bGH treatment on plasma concentrations of GH (ng/ml) on day 4 and 5 of treatment (Means for day 4 and day 5 are covariate-adjusted to day -1 values). Values are presented as mean±SE

Age	Dose of bGH	Day 4+Day 5	
(days)	(mg/kg LW/day)	Saline	bGH
1-7	0.15	25.0 ± 5.9^{b}	18.5 ± 6.1^{b}
14-21	0.15	21.4 ± 5.9^{b}	34.1 ± 5.9^{bc}
28-35	0.15	26.7 ± 5.9^{bc}	42.4 ± 6.1^{c}
56-63	0.15	27.3 ± 6.2^{bc}	28.7 ± 5.8^{bc}
365	0.10	4.8 ± 4.2^{a}	29.5 ± 4.2^{bc}
365	0.20		37.9 ± 4.2^{c}

^{abc} means with different superscripts are significantly different ($P<0.05$)

Table 6.2 Effects of age and bGH treatment on plasma concentrations of IGF-I (ng/ml) on day 4 and 5 of treatment (Means for day 4 and day 5 are covariate-adjusted to day -1 values). Values are presented as mean±SE

Age	Dose of bGH	Day 4+Day 5	
(days)	(mg/kg LW/day)	Saline	bGH
1-7	0.15	185.6±11.3^{b}	256.8±10.7^{d}
14-21	0.15	150.5±10.8^{b}	200.5±10.7^{c}
28-35	0.15	163.8±10.9^{b}	225.6±12.2^{c}
56-63	0.15	169.3±10.7^{b}	232.1±10.7^{c}
365	0.10	76.6±10.8^{a}	201.8±10.7^{c}
365	0.20		259.1±10.8^{d}

^{abcd} means with different superscripts are significantly different ($P<0.05$)

Effects of age and GH treatment on circulating concentrations of insulin at the end of treatment are presented in Table 6.3. In lambs, plasma concentrations of insulin in saline-treated animals decreased abruptly from 1799.2±363.0 pg/ml at day 6/7 of life to 771.4±273.5 pg/ml at day 20/21 of life and remained stable thereafter. A similar trend was also apparent in bGH-treated animals. As a result, there was significant effect of age on plasma concentrations of insulin across treatment groups. GH treatment had no effect on plasma concentrations of insulin except in the 14-21 day group, and in yearling sheep, where GH treatment increased circulating concentrations of insulin.

Table 6.3 Effects of age and bGH treatment on plasma concentrations of insulin (pg/ml) on day 4 and 5 of treatment (Means for day 4 and day 5 are covariate-adjusted to day -1 values). Values are presented as mean±SE

Age	Dose of bGH	Day 4+Day 5	
(days)	(mg/kg LW/day)	Saline	bGH
1-7	0.15	1799.2±363.0^{b}	1974.3±304.0^{b}
14-21	0.15	771.4±273.5^{a}	1411.6±271.01^{b}
28-35	0.15	968.1±279.3^{a}	908.3±291.4^{a}
56-63	0.15	850.7±294.9^{a}	902.2±280.6^{a}
365	0.10	758.1±444.6^{a}	2015.8±444.0^{b}
365	0.20		2898.7±444.6^{c}

^{abc} means with different superscripts are significantly different ($P<0.05$)

6.4.2 Plasma concentrations of glucose, NEFA and urea

Circulating concentrations of glucose were high at day 6/7 of life but decreased with increasing age in both groups (Table 6.4). As a result, there was a significant effect of age on plasma glucose levels across treatment groups. GH treatment had no effect on circulating concentrations of glucose in lambs up to day 63 of life, whereas it significantly increased the concentration in yearling sheep at both doses.

Table 6.4. Effects of age and bGH treatment on plasma concentrations of glucose (mM) on day 4 and 5 of treatment (Means for day 4 and day 5 are covariate-adjusted to day -1 values). Values are presented as mean±SE

Age	Dose of bGH	Day 4+Day 5	
(days)	(mg/kg LW/day)	Saline	bGH
1-7	0.15	5.51±0.15^{d}	5.75±0.15^{d}
14-21	0.15	5.14±0.15^{c}	5.26±0.16^{c}
28-35	0.15	5.32±0.15^{cd}	5.13±0.15^{c}
56-63	0.15	4.94±0.15^{c}	5.07±0.15^{c}
365	0.10	3.64±0.11^{a}	4.02±0.11^{b}
365	0.20		4.13±0.11^{b}

^{abcd} means with different superscripts are significantly different ($P<0.05$)

Circulating concentrations of NEFA in saline-treated animals were not different in lambs up to day 63 of life although they were much higher in lambs than in yearling sheep (Table 6.5). However, plasma concentrations of NEFA in GH-treated lambs tended to increased with age, as evidenced by a significant ($P<0.01$) interaction between treatment and age. As a result, plasma concentrations of NEFA at day 62/63 of life were significantly ($P<0.05$) higher in bGH-treated lambs than in saline-treated lambs. In yearling sheep, exogenous GH stimulated circulating concentrations of NEFA at both doses, although absolute levels were lower than in lambs.

Table 6.5 Effects of age and bGH treatment on plasma concentrations of NEFA (mEq/l) on day 4 and 5 of treatment (Means for day 4 and day 5 are covariate-adjusted to day -1 values). Values are presented as mean±SE

Age	Dose of bGH	Day 4+Day 5	
(days)	(mg/kg LW/day)	Saline	bGH
1-7	0.15	0.299±0.038^{c}	0.348±0.038^{c}
14-17	0.15	0.286±0.038^{c}	0.300±0.038^{c}
28-35	0.15	0.389±0.038^{c}	0.450±0.040^{cd}
56-63	0.15	0.283±0.038^{c}	0.552±0.038^{d}
365	0.10	0.078±0.025^{a}	0.138±0.023^{b}
365	0.20		0.180±0.024^{b}

^{abcd} means with different superscripts are significantly different ($P<0.05$)

Plasma concentrations of urea at day 4 and 5 of treatment are presented in Table 6.6. In young lambs, circulating concentrations of urea decreased from day 6/7 of life to day 34/35 of life and increased thereafter. In general, GH treatment had no effect on circulating concentrations of urea at any age, including yearling sheep (with the exception of a small, but significant, reduction in the yearlings treated with high dose bGH).

Table 6.6 Effects of age and bGH treatment on plasma concentrations of urea (mM) on day 4 and 5 of treatment (Means for day 4 and day 5 are covariate-adjusted to day -1 values). Values are presented as mean±SE

Age	Dose of bGH	Day 4+Day 5	
(days)	(mg/kg LW/day)	Saline	bGH
1-7	0.15	8.4±0.6^{ab}	7.2±0.5^{a}
14-17	0.15	6.8±0.5^{a}	6.5±0.5^{a}
28-35	0.15	7.3±0.5^{ab}	6.6±0.5^{a}
56-63	0.15	8.3±0.5^{ab}	7.6±0.5^{ab}
365	0.10	8.9±0.2^{c}	8.5±0.2^{c}
365	0.20		8.3±0.2^{b}

^{abc} means with different superscripts are significantly different ($P<0.05$)

6.4.3 Hepatic GHR measurements

Hepatic GHR were measured only at days 7 and 63 of life, and in yearlings. Hepatic GHR concentrations in the control group were similar across ages (Table 6.7). bGH treatment had no significant effect on hepatic GHR levels at days 7 and 63 of life. By contrast, in yearling sheep, GH treatment at the low dose (0.1 mg/kg LW) significantly ($P<0.05$) increased hepatic GHR with a further increase at the high dose (0.2 mg/kg LW).

Table 6.7 Effects of age and bGH treatment on specific binding (SB) of ^{125}I-labelled oGH to hepatic membrane preparations (mean±SE)

Age	Dose of bGH	^{125}I-oGH binding (%SB/mg protein)	
(days)	(mg/kg LW/day)	Saline	bGH
7	0.15	2.43±0.19[a]	2.60±0.16[a]
63	0.15	2.86±0.20[a]	2.58±0.18[a]
365	0.10	2.57±0.13[a]	3.02±0.12[b]
365	0.20		3.15±0.12[b]

[ab] means with different superscripts are significantly different ($P<0.05$)

6.5 DISCUSSION

It has been shown previously that lambs less than three weeks old do not respond to exogenous GH by increasing circulating insulin-like growth factor-I (IGF-I) concentrations (Chapter 4). A similar result has been also reported in other neonatal sheep (S. N. McCutcheon, personal communication), in which chronic treatment of GH had little effect on circulating concentrations of IGF-I. These results are in contrast to those in older sheep (Pell *et al.* 1990; McLaughlin *et al.* 1993*a,b*) in which GH treatment has a stimulatory effect on circulating concentrations of IGF-I. Although young lambs are known to exhibit adult-like binding of GH to hepatic receptors (Gluckman *et al.* 1983), the inability of young lambs to secrete IGF-I in response to exogenous GH stimuli suggests that hepatic GHR are present but non-functional (or not fully functional).

In this study, exogenous GH had no effect on circulating concentrations of GH in young lambs up to 63 days of life although it tended to increase plasma GH levels in lambs at days 20/21 and 34/35 of life. In contrast, administration of GH at the low dose (0.1 mg/kg) increased circulating concentrations of GH up to six-fold in yearling sheep

with a further increase at the high dose (0.2 mg/kg). The reason why exogenous GH apparently failed to increase plasma GH levels in young lambs remains unclear, but it may be partly associated with the blood sampling regimen employed in the present study. Blood samples were taken only once daily, 7.5 h after GH injection, in both young and old sheep. This was done to minimize handling of the young lambs and the risk of mismothering which could have led to depressed milk intakes in the lambs and a suppression of hepatic GH receptors (Breier *et al.* 1988*a*; Bass *et al.* 1991). A retrospective analysis of GH concentrations in samples taken at 3, 7 and 15 h post-GH injection in the previous study (Chapter 4) also suggested that differences in plasma GH concentrations between saline- and GH-treated lambs had diminished by 7 h after injection. These observations suggest that clearance of GH from plasma may be much faster in young lambs than in yearlings. Thus in future studies it may be necessary to employ more intensive blood sampling regimens despite the risk of disturbing the dam-offspring bond, especially in very young lambs, or to use lambs bottle-fed from birth (as in Chapter 4).

The suggestion that the lack of an effect of exogenous GH on circulating concentrations of GH in young lambs was associated with the blood sampling regimen, rather than with failure of the treatment protocol, is further supported by the observation that GH treatment significantly stimulated circulating concentrations of IGF-I in all groups of young lambs. The extent of the plasma IGF-I response to exogenous GH was similar among lambs up to 63 days of life, ranging from 33-38% of control levels at each age. However, the IGF-I response in young lambs was much lower than that observed in yearling sheep, where GH treatment caused plasma IGF-I levels to increase 3-fold. These results are consistent with the previous study (Chapter 4), in which GH treatment of lambs for 21 days from day 3 of life had only a small effect on circulating concentrations of IGF-I. However, the extent of the plasma IGF-I response to a GH stimulus was smaller (13% of control levels) in the previous study (Chapter 4) than that observed in the present study, possibly because of administration of the lower dose of GH (0.1 mg/kg LW) in the former experiment. A similar result has been demonstrated also in neonatal cattle (Groenewegen *et al.* 1990), where chronic treatment with GH failed to affect plasma concentrations of IGF-I.

It is well recognized that the appearance of hepatic GHRs is important for IGF-I

production and postnatal growth in sheep and cattle. In sheep, hepatic GHRs appear within days of birth and receptor concentrations reach adult values very rapidly (Gluckman *et al.* 1983). Such a finding is further demonstrated in the present study, in which (unstimulated) hepatic GHR concentrations in young lambs were similar to those in yearling sheep. Despite similar levels of hepatic GHR between lambs and yearling sheep, the magnitude of the IGF-I response to exogenous GH in young lambs was small compared to that observed in the yearlings. This result suggests that hepatic GHRs may not be fully functional in young lambs. Furthermore, the "degree of functionality", as measured by the IGF-I response to GH, does not appear to alter greatly over the first 9 weeks of life. Rather, the major ontogenetic change must occur between 9 weeks and the yearling stage. Consistent with this, Roberts *et al.* (1990), who studied the ontogeny of IGF-I in intact sheep from about 3 months of age to 16 months of age, reported that circulating concentrations of IGF-I increased markedly from day 238 of life (when sheep reached puberty) and peaked around day 321 of life. The concentrations then remained constant or declined slightly. The rise in plasma IGF-I concentrations at puberty may be related to a rise in circulating sex steroids since plasma IGF-I levels are increased by treatment with physiological doses of estrogen (Breier *et al.* 1988*b*) and androgen (Jasper, 1985). It is thus conceivable that puberty alters functionality of the GHR. A similar ontogeny is seen with respect to glucose and insulin responses to GH, which do not occur in young lambs but are evident in yearlings.

By contrast, a somewhat different ontogeny was evident with respect to the effects of GH on lipolysis. Thus a significant effect of GH on circulating NEFA concentrations was first observed at day 62/63 and the yearling response was proportionally similar (an approximate doubling of plasma NEFA concentrations, averaged across the two GH doses). This may reflect the direct effect of GH on adipose tissue (Vernon, 1989) and imply different patterns of maturation of GH receptors in the liver versus the adipose tissue. Alternatively, the ability of GH to stimulate NEFA at day 62/63 may reflect change from glucose-based energy metabolism (pre-ruminant phase) to a situation where metabolism of volatile fatty acids produces a greater proportion of the energy (ie the change to a fat-based metabolism), as lambs consume a considerable amount of solid food at this stage (Doney, 1982/1983).

The suggestion that hepatic GHRs may not be fully functional in young lambs is

further supported by the observation that exogenous GH failed to alter hepatic GHR number in young animals. In contrast, in yearling sheep, GH treatment increased hepatic GHRs in a dose-dependent manner. Such an up-regulation of hepatic GH binding sites in yearling sheep, but not in young lambs, may provide a possible explanation for different IGF-I responses between two groups. It is well established that specific binding of GH to hepatic membranes involves high and low affinity binding sites in a number of species, including sheep (Pell *et al.* 1990; Sauerwein *et al.* 1991). Sauerwein *et al.* (1991) reported from studies with well-fed sheep that GH treatment largely up-regulated high affinity GHR with little effect on low-affinity binding sites. Therefore, it is likely that the up-regulation of GHR concentration by exogenous GH observed in yearling sheep largely reflects changes in high affinity GHR. Although such an up-regulation of hepatic GHR was not demonstrated in young lambs, the positive response of plasma IGF-I to exogenous GH stimulus in young lambs raises the possibility that GH treatment may also lead to a greater proportion of high binding sites with no change in GHR number. This may be responsible for a significant increase in plasma IGF-I levels in young lambs, despite the inability of GH to affect the GHR in the same group. Whatever the mechanisms, the present result, together the corresponding change in plasma IGF-I concentrations between young and yearling sheep, suggests that the GH-IGF-I axis, unlike that in yearlings, is not fully developed in young lambs. Such a slow development of the GH-IGF-I axis in young animals provides a possible explanation for the lack of a growth-promoting effect of GH observed in previous studies (Sun *et al.* 1991; Chapter 4). This situation is apparently different from that in cattle, where the low level of hepatic GHR (Badinga *et al.* 1991), as opposed to limited GHR functionality, is considered to be the major factor responsible for the delayed growth response to GH observed in neonatal calves (Groenewegen *et al.* 1990). Therefore, there appears to be a marked distinction between sheep and cattle with regard to the ontogeny of hepatic GHR.

In conclusion, the present data showed that GH treatment had a small effect on circulating concentrations of IGF-I in young lambs. This observation, together with the corresponding change in hepatic GHR, suggests that hepatic GHR may not be fully functional at this stage.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Placental lactogens are a member of the hormone family which also includes GHs and PRLs. PL is secreted by the placenta and has been implicated in the regulation of fetal growth and mammogenesis. The presence of PLs was first noted in the early 1930s when research into GHs commenced (Rand-Weaver *et al.* 1993). Nevertheless, progress in understanding the biological role of PL in farm animal production has been very limited compared to the situation with GH. This has been partly due to the fact that characterization of the full amino acid sequence of PLs, including those of ruminants, has not occurred until recent years.

The amino acid sequence of PLs in farm animals was first determined in cattle (Schuler *et al.* 1988) and subsequently in sheep (Colosi *et al.*1989; Warren *et al.* 1990*b*). This characterization of the amino acid sequence of ruminant PLs has led to the production of biosynthetic PL. Several groups have used this opportunity to examine the biological actions of PL. Results have shown that ruminant PLs exhibit both lactogenic (Vega *et al.* 1989; Byatt *et al.* 1992*a*, 1994) and somatogenic (Byatt *et al.* 1991; Singh *et al.* 1992) activities *in vivo*, consistent with their structural similarity to PRLs and GHs (Warren *et al.* 1990*b*). While these data, together the results from earlier studies (Hurley *et al.* 1977; Servely *et al.* 1983; Byatt & Bremel, 1986), raise the possibility that PLs have potential use in manipulating farm animal production, many of these studies have been conducted using heterologous systems. Furthermore, animals in some of the *in vivo* studies were GH-deficient strains (Hurley *et al.* 1977; Singh *et al.* 1992), which causes a further complication in interpreting some of the results. A similar situation exists with regard to IGF-I, its biological actions having been assessed largely in GH-deficient animals rather than in animals with an intact somatotrophic axis. Therefore, the objective of this study was to examine the biological actions of recombinant oPL and IGF-I in intact animals and to evaluate the their potential use to stimulate performance of farm animals.

It is well recognized that exogenous GH stimulates milk yields in lactating animals,

including sheep and cattle (Bauman & McCutcheon, 1986). Such a galactopoietic effect of GH was observed in the present study (Chapter 3), consistent with previous findings. There is also some evidence that bGH treatment during late gestation stimulates mammary gland development in cattle (Stelwagen *et al.* 1992) and sheep (Kann *et al.* 1988) although the enhanced mammogenesis following GH treatment does not necessarily lead to increased milk yields in the subsequent lactation period (Simpson *et al.* 1992). However, in the present study (Chapter 2), bGH treatment for seven days beginning on day 101 of gestation did not affect mammary gland development in pregnant ewes. The inability of bGH to stimulate mammogenesis in the present study, in contrast to that observed in previous studies, may be associated with the short treatment period since, in previous studies, GH treatment lasted for minimum of 10 days. This suggestion is further supported by recent studies with pregnant ewes (C. M. C. Jenkinson, D. D. S. Mackenzie and S. N. McCutcheon, personal communication), in which bGH administration at the same dose for 14 days beginning on days 70 or 98 of gestation enhanced mammary gland development as measured by dimension or trimmed weight of the mammary gland.

Administration of bGH to pregnant ewes also increased total uterine weight in the present study (Chapter 2). Although the increase in total uterine weight was associated mainly with a significant increase in weights of uterine fluids, fetal weight also tended to be greater in bGH-treated dams than in saline-treated animals. A similar result has been obtained in recent studies with pregnant ewes (C. M. C. Jenkinson, D. D. S. Mackenzie and S. N. McCutcheon, personal communication), in which treatment with bGH at the same dose as used in the present study for 14 days beginning on day 98 of gestation significantly increased the total uterine weight as well as fetal weight. These data suggest that administration of GH to pregnant ewes during late gestation may have beneficial effects on fetal growth. Such a stimulatory effect of GH on fetal weight has been reported also in the pig (Kelly *et al.* 1995; Sterle *et al.* 1995), although the effect was most apparent in the early stages of gestation.

While GH treatment in the late gestation and early lactation periods stimulated uterine development and milk yield respectively, no such effects were apparent in oPL-treated sheep (Chapters 2 and 3). Similarly, oPL treatment in late gestation failed to affect mammary gland development (Chapter 2). It is well recognized that IGF-I plays

an important role in mediating many of the biological actions of GH, probably including galactopoiesis. Measurement of plasma IGF-I concentrations in the present studies showed that bGH treatment led to substantial increases in circulating concentrations of IGF-I, suggesting that the same mechanisms may be responsible for the increased total uterine weight and milk yield (Chapters 2 and 3). In contrast, plasma IGF-I concentrations were not affected by oPL treatment. Therefore, the failure of exogenous oPL to have biological actions in pregnant and lactating ewes may have been associated with its inability to alter circulating concentrations of IGF-I. Whatever the reasons, the present results clearly suggests that the mechanisms by which oPL acts are different from those of GH.

Although the present studies failed to demonstrate any biological actions of oPL in pregnant and lactating ewes, these data do not necessarily exclude the possibility that this hormone is biologically active, particularly during gestation. oPL has been consistently implicated in the regulation of fetal growth (Gluckman *et al.* 1979*b*; Taylor *et al.* 1980; Thordarson *et al.* 1987; Kappes *et al.* 1992) and mammogenesis (Butler *et al.* 1981; Schams *et al.* 1984). In the present study (Chapter 2), circulating concentrations of oPL prior to treatment were positively correlated with fetal weight, suggesting a positive role of PL in the regulation of fetal growth. The failure of the present study to detect any GH-like activity of oPL may be partly associated with the dose employed. For example, administration of bGH to pregnant ewes (Chapter 2) raised plasma GH levels 9-fold, while administration of the same dose of oPL raised oPL levels only 1.5-fold. These results suggest that oPL was absorbed more slowly from the injection site than bGH or, more likely, that it is cleared rapidly from circulation compared with bGH. Under this situation, the dose of oPL employed in the present studies (Chapters 2 and 3) may not have been sufficiently high to cause biological actions. However, in lactating cattle, recombinant bPL administered at a similar dose has been shown to have galactopoietic effects although those lactational studies, unlike the present study, were conducted during mid- to late-lactation (Byatt *et al.* 1992*a*).

Alternatively, the lack of biological actions of oPL (Chapters 2 and 3) may have been due partly to the short length of the treatment period. In the present studies, effects of exogenous oPL were measured over periods of only 5 and 7 days, mainly because of the limited supply of recombinant oPL available. By contrast, in studies with dairy cattle

in which recombinant bPL was shown to be biologically active, bPL treatment lasted for either 9 or 14 days (Byatt *et al.* 1992*a*, 1994). It is impossible to make direct comparisons between the bPL studies and the present experiments (Chapters 2 and 3) because the hormonal environment of animals used in the two situations was likely to have been quite different. Nevertheless, the positive effects of recombinant bPL observed in the previous studies raise the possibility that the length of treatment period employed here may not have been long enough to elicit biological activities.

The importance of the length of treatment period for eliciting biological actions of oPL is further demonstrated by the subsequent study (Chapter 4), in which somatogenic actions of recombinant oPL in milk-fed lambs were observed when treatment continued over a prolonged period (21 days from day 3 of life). Thus administration of oPL significantly increased the liveweights of lambs from about day 10 after the commencement of treatment. Such increases in the growth rates of oPL-treated lambs were accompanied by corresponding increases in voluntary feed intake, an effect not previously reported in sheep.

The acute effect of oPL on voluntary feed intake is interesting since the increases in feed intake following GH treatment occur only after several weeks of continuous treatment (Bauman *et al.* 1985; Peel *et al.* 1985; Sandles *et al.* 1988). Such acute responses to PL in voluntary feed intake have also been reported in studies with rats (Byatt *et al.* 1991) and cattle (Byatt *et al.* 1992*a*), where administration of recombinant bPL stimulated voluntary feed intake within several days of the commencement of treatment. Thus the acute response in voluntary feed intake appears to be a common characteristic of ruminant PL across species. The mechanisms by which PL stimulates feed intake remains unclear although, in the rat, the PRL receptor is implicated in this process (Byatt *et al.* 1991). Such an acute increase in voluntary feed intake following PL treatment appears likely to be an important contributor to the enhanced growth rate observed in the present experiment (Chapter 4), whereas in studies with bPL the response in voluntary feed intake tends to be confounded with a response in plasma IGF-I concentrations.

While exogenous oPL treatment stimulated both the growth rate and voluntary feed intake of milk-fed lambs, administration of the same dose of GH had no effect on either parameter (Chapter 4). A similar result has been reported in another study with neonatal

lambs (Sun *et al.* 1991), in which chronic treatment with bGH from the day of birth to 11 weeks of age failed to alter growth rate. Although newborn lambs are reported to exhibit adult-like binding of GH to hepatic receptors (Gluckman *et al.* 1983), exogenous GH had little or no effect on circulating concentrations of IGF-I in newborn lambs (Chapter 4; S. N. McCutcheon, personal communication). These results are in contrast to those in older sheep (Pell *et al.* 1990; McLaughlin *et al.* 1993*a, b*; Chapters 2 and 3) in which GH treatment had a stimulatory effect on circulating concentrations of IGF-I. The inability of young lambs to secrete IGF-I in response to exogenous GH stimuli suggests that hepatic GHR may exist but not be fully functional. This hypothesis was examined by the subsequent study (Chapter 6), in which the ontogeny of hepatic GHR was assessed by measuring the plasma IGF-I and hepatic GHR response to a fixed dose of bGH at days 6-7, 20-21, 34-35 and 62-63 of life, and in yearlings. Results showed that the plasma IGF-I response to exogenous GH in young lambs up to day 63 of life was small compared to that observed in yearling sheep (which achieved similar plasma concentrations of IGF-I following GH stimulation but from a much lower baseline). Furthermore, the degree of "GH receptor functionality", as measured by the IGF-I response to GH, does not appear to alter greatly over the first 9 weeks of life, suggesting that the major ontogenic changes must occur between 9 weeks and the yearling stage. Consistent with this, exogenous GH failed to affect hepatic GHRs in young lambs, but up-regulated them in yearling sheep. These results suggest that the GH-IGF-I axis is not fully developed in young lambs compared with the situation in older sheep. Such an uncoupling of GH-IGF-I in young lambs may, therefore, be responsible for the lack of a growth response to GH observed in earlier studies with young lambs (Sun *et al.* 1991; Chapter 4).

The lack of a growth response following GH treatment, and the lack of a change in plasma IGF-I concentrations, in the present study (Chapter 4) provides further evidence that circulating concentrations of IGF-I are an important mediator of the somatogenic actions of GH. This suggestion is consistent with previous reports that systemically administered IGF-I stimulates growth in a number of situations (Robinson & Clark, 1989). However, such growth-promoting effects of IGF-I have been observed mainly in GH-deficient animals such as hypophysectomized or dwarf rats. In contrast, administration of exogenous IGF-I has little or no effect on growth in normal growing

animals with an intact somatotrophic axis (Guler *et al.* 1989*a*; Cottam *et al.* 1992). It has been suggested that the failure of IGF-I to promote growth in well-fed intact animals may be associated with their already high plasma levels of IGF-I (Schalch *et al.* 1989; Young *et al.* 1990). However, in the present study with sheep (Chapter 5), in which low control plasma concentrations of IGF-I were ensured by the restriction of dietary energy intake, chronic treatment with IGF-I failed to exhibit growth-promoting effects. Measurement of plasma GH and hepatic GHR showed that exogenous IGF-I suppressed circulating concentrations of GH and also down-regulated hepatic GHR. These findings, not previously reported, suggest that the lack of growth-promoting effects of IGF-I in the present study and other studies (Cottam *et al.* 1992) may be in part due to negative feedback effects on both circulating concentrations of GH and hepatic GH receptor numbers. Therefore, the potential of IGF-I to enhance performance of intact animals appears to be limited by its negative feedback regulation of the somatotrophic axis.

While exogenous IGF-I failed to have growth-promoting effects in underfed sheep (Chapter 5), it increased weights of the spleen and thymus as well as eosinophil numbers. A similar result has been reported in another study with normal growing rats (Clark *et al.* 1993), in which IGF-I treatment caused substantial increases in several immune parameters, including leucocyte numbers. These data suggest that IGF-I may have beneficial effects on animal performance by enhancing immune function.

Finally, it is important to consider the practical implications of these studies, which may be summarised as follows;

1. Although not central to the research programme, the observation (Chapter 2) that bGH treatment improves uterine development and perhaps fetal growth could be relevant to attempts to control birth weights of farm animal species. For example, low birth weight of multiple-born lambs is a significant factor contributing to their high mortality rates (Dalton *et al.* 1980; Hinch *et al.* 1983). Thus the present study and those which have followed (C. M. C. Jenkinson, D. D. S. Mackenzie and S. N. McCutcheon, personal communication) may provide a means of improving fetal growth and reducing mortality through the use of GH therapy.

2. Further studies are required to examine the effects of oPL in pregnant and lactating ewes but they will need to be of longer duration than those conducted here. This is particularly true with regard to galactopoiesis since such effects have been demonstrated from bPL treatment of lactating cattle (Byatt *et al.* 1992*a*). Given that GH and PL appear to stimulate lactational performance by different means, the question also arises whether their effects might be additive.

3. The observation (Chapter 4) that oPL treatment has growth-promoting effects via its stimulation of feed intake potentially provides a new means to enhance the growth of farm animals. For example, in situations such as neonatal animals (as demonstrated in Chapter 4), where GH has no growth-promoting effects because of immature hepatic GHRs, oPL treatment could provide an alternative way to enhance growth rate. The potential benefit of the application of oPL to the young animal is further illustrated by the fact that the advantage in liveweight following oPL treatment was still maintained 250 days after termination of treatment. This may imply that early life oPL treatment somehow "re-sets" endogenous limits to growth.

4. oPL treatment may also have specific application to pasture production systems. In those systems, which are characterised by highly variable feed supply through the season, the ability to stimulate appetence may have a unique value because it permits the regulation of herbage accumulation and hence quality. Thus oPL could have application as an "intake stimulant" at times (eg in the spring) when pasture growth rate typically exceeds the ability of animals to consume it.

5. The failure of IGF-I to enhance growth in both well-fed (Cottam *et al.* 1992) and under-fed (Chapter 5) sheep suggests that IGF-I is unlikely to be useful as a growth promotant in intact animals because of its down-regulation of the somatotrophic axis. However, in young lambs (Chapter 4 and 6), down-regulation of the hepatic GHR by IGF-I, as occurs in the adult, may not be an issue because the GHR are at that time not fully functional. Hence IGF-I could have potential as a growth promotant in young lambs, and more generally in the young of other

species.

6. Since oPL does appear to promote growth in young lambs, but not via changes in plasma IGF-I concentrations, there is the possibility that oPL and IGF-I could have additive effects in young animals. This possibility reflects the fact that, in the young growing animal, oPL does not appear to act by pathways associated with the somatotrophic axis.

In conclusion, these studies suggest that, if oPL and IGF-I have a role in the enhancement of farm animal performance, that role is most likely to apply in the improvement of growth rate in the young animal.

APPENDIX

LIST OF PUBLICATIONS

The following publications arose directly from this thesis (*) or from the associated research programme.

1. Klempt M, Breier BH, **Min SH**, Mackenzie DDS, McCutcheon SN & Gluckman PD 1993 IGFBP-2 expression in liver and mammary tissue in lactating and pregnant ewes. *Acta Endocrinologica* **129** 453-457.
2. Currie MJ, Bassett NS, Breier BH, **Min SH**, Mackenzie DDS, McCutcheon SN & Gluckman PD 1993 Ovine placental lactogen is not galactopoietic in the lactating sheep. *Proceedings of the 36th Annual Meeting of the Endocrine Society of Australia.* Abstract No. 39.

*3. **Min SH**, Mackenzie DDS, Breier BH, McCutcheon SN & Gluckman PD 1994 Growth-promoting and metabolic actions of recombinant ovine placental lactogen and bovine growth hormone in young lambs. *Proceedings of the New Zealand Society of Animal Production* **54** 59-62.

*4. **Min SH**, Mackenzie DDS, Breier BH, McCutcheon SN & Gluckman PD 1994 Anabolic effects of chronic IGF-I administration in underfed yearling sheep. *Proceedings of the 37th Annual Meeting of the Endocrine Society of Australia.* Abstract No. NZ8.

5. Currie JM, Breier BH, **Min SH**, McCutcheon SN, Mackenzie DDS & Bassett NS 1994 Ovine placental lactogen increases IGFBP-3 gene expression. *Third International Symposium on Insulin-like Growth Factors, Sydney, Australia.* Abstract No. 115.

*6. **Min SH**, Mackenzie DDS, Breier BH, McCutcheon SN & Gluckman PD 1995 Responses of young energy-restricted sheep to chronically administered IGF-I: evidence that IGF-I suppresses the hepatic growth hormone receptor. *Endocrinology* (In Press).

*7. **Min SH**, Mackenzie DDS, Breier BH, McCutcheon SN & Gluckman PD 1995 The effects of recombinant ovine placental lactogen (oPL) in young lambs: comparison with bovine growth hormone provides evidence for a distinct effect of oPL on food

intake. *Growth Regulation* (In Press).

8. Currie MJ, Bassett NS, Breier BH, Klempt M, **Min SH**, Mackenzie DDS, McCutcheon SN & Gluckman PD 1995 Differential effects of maternal ovine placental lactogen (oPL) and growth hormone (GH) administration on GH receptors, insulin-like growth factor (IGF)-1 and IGF binding protein-3 gene expression in the pregnant and fetal sheep. *Growth Regulation* (In Press).

*9. **Min SH**, Mackenzie DDS, Breier BH, McCutcheon SN & Gluckman PD 1995 Recombinant ovine placental lactogen and bovine growth hormone have differential effects on galactopoiesis in ewes. *Journal of Dairy Science* (Submitted).

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