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THE REGULATION OF POST-NATAL GROWTH IN SHEEP THROUGH THE GROWTH HORMONE - INSULIN-LIKE GROWTH FACTOR AXIS

A thesis
submitted in fulfilment
of the requirements for the Degree
of
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JENNY MIREE OLDHAM

University of Waikato

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For Colin and Jackson.

ABSTRACT

The GH-IGF axis is one hormonal system which is considered to control muscle growth. To investigate the factors controlling post-natal growth of skeletal muscle in lambs, methods of histological autoradiography were developed in the experiments presented in this thesis to provide sensitive measurement of hormone binding *in vitro* to receptors. The techniques were used to measure receptors for GH, IGF1 and IGF2 in skeletal muscle of prepubertal lambs which had undergone treatment with exogenous growth factors and/or degrees of nutritional restriction before slaughter.

Plasma concentrations of IGF1 were positively correlated with good nutrition and treatment of animals with exogenous GH. Whether or not the changes in plasma IGF1 concentrations are associated with changes in the binding of IGF1 to skeletal muscle is possibly dependent on the age of the animal, the severity of nutritional restriction and the tissue type in skeletal muscle from which measurements were taken. Binding of IGF1 was demonstrated in both the muscle fibre and connective tissue in lambs of six months old, with an increase of binding in connective tissue associated with fasting. In lambs of eight months old, binding was present only in connective tissue and not regulated by GH treatment or nutritional restriction of animals.

Plasma concentrations of IGF2 were not altered with fasting of animals. Binding of IGF2 was restricted in distribution to connective tissue in six month old lambs and also insensitive to fasting. The greatest density of IGF1 and IGF2 binding in skeletal muscle was always observed associated with blood vessels. GH binding in skeletal muscle was not demonstrated.

In conclusion, connective tissue appears to be a more sensitive target tissue than muscle fibre through which the IGFs may mediate the effects of changes in nutritional status.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
b	bovine
BSA	bovine serum albumin
BST	bovine somatotropin
cpm	counts per minute
FITC	fluorescein isothiocyanate
GH	growth hormone
GRH	growth hormone releasing hormone
h	human
IGF	insulin-like growth factor
IgG	immunoglobulin
N-Met	N-terminus methionyl
o	ovine
PBS	phosphate buffered saline
PBSA	phosphate buffer with BSA
PEG	polyethylene glycol
PMSF	phenylmethanesulphonyl fluoride
SRIF	somatostatin
T4	thyroxine
TBS	tris buffered saline
TRH	thyrotropin releasing hormone

CHAPTER ONE:

INTRODUCTION

It has become increasingly apparent over recent decades that a complex and integrated process such as growth is not solely controlled by any one variable. In addition to the hormones which are involved, there are environmental and nutritional considerations and increasing evidence that the target tissues participate in regulating growth (Spencer, 1985). Growth hormone (GH) is a central endocrine stimulus of normal growth in mammals. Removal of endogenous GH by hypophysectomy severely retards growth, while normal growth is largely restored with GH therapy (Bass and Clark, 1989). The demonstration of this association in numerous species has arisen from a huge area of clinical research attempting to understand growth disorders and the interaction between nutrition, GH and growth in humans, and also to studies in which the primary interest is the manipulation of post-natal growth in agricultural species.

THEORIES OF GROWTH HORMONE ACTION

The somatomedin hypothesis of growth, originally postulated by Salmon and Daughaday (1957) proposes that the growth effects of GH on peripheral tissues are mediated by a second group of endocrine factors. Originally known as sulphation factors, non-suppressible insulin-like activity or somatomedins, they have been more recently termed the insulin-like growth factors (IGFs) (Daughaday, Hall, Salmon *et al.*, 1987). It was suggested that IGFs in plasma, predominantly IGF1 secreted by the liver in response to GH stimulation (Schwander, Hauri, Zapf *et al.*, 1983), were transported to peripheral tissues where they stimulated growth. This explanation has been confounded by the finding that most, if not all, tissues are capable of synthesizing IGFs (Romanus, Yang,

Adams *et al.*, 1988) and that stimulation of IGF production in peripheral tissues can precede elevated concentrations in plasma (D'Ercole, Stiles and Underwood, 1984). Whether IGFs are effective in an endocrine or paracrine manner is not necessarily mutually exclusive. Daughaday (1989) considers that both actions occur, with the relative importance of each depending on numerous factors.

More recent theories on the control of growth by GH contrast with the sequential nature of GH stimulation via IGFs as proposed by the somatomedin hypothesis. The dual effector theory (Green, Morikawa and Nixon, 1985) and differential clonal expansion theory (Isaksson, Lindahl, Nilsson *et al.*, 1987) both propose that GH and IGF1 act in a coordinated manner on the same target cells to induce growth. This is supported by the demonstration of a much wider distribution of GH receptors than previously thought (Lobie, Breipohl and Waters, 1990b) and in this context GH is considered to be a differentiating factor which sensitizes cells to the mitogenic action of IGFs (Green *et al.*, 1985; Isaksson *et al.*, 1987). Further, there is evidence that IGFs are secreted by peripheral target tissues in response to GH stimulation (Isgaard, Nilsson, Lindahl *et al.*, 1986), possibly for paracrine or autocrine action on cells. The support of normal growth by peripheral production of IGFs has not yet been demonstrated (Daughaday, 1989), although IGF1 replacement therapy in hypophysectomised animals does stimulate growth (Schoenle, Zapf, Humbel *et al.*, 1982). Possible routes of action for the effects of GH and IGF1 on growth are portrayed in Figure 1.1. GH may directly stimulate growth in peripheral tissues, act via stimulation of IGFs to be transported in plasma (endocrine action of IGFs) or by stimulation of IGFs in peripheral tissues (paracrine or autocrine action of IGFs).

In the review which follows, characteristics of GH and the IGFs in relation to growth and development are presented independently and, wherever possible, with particular reference to ruminants.

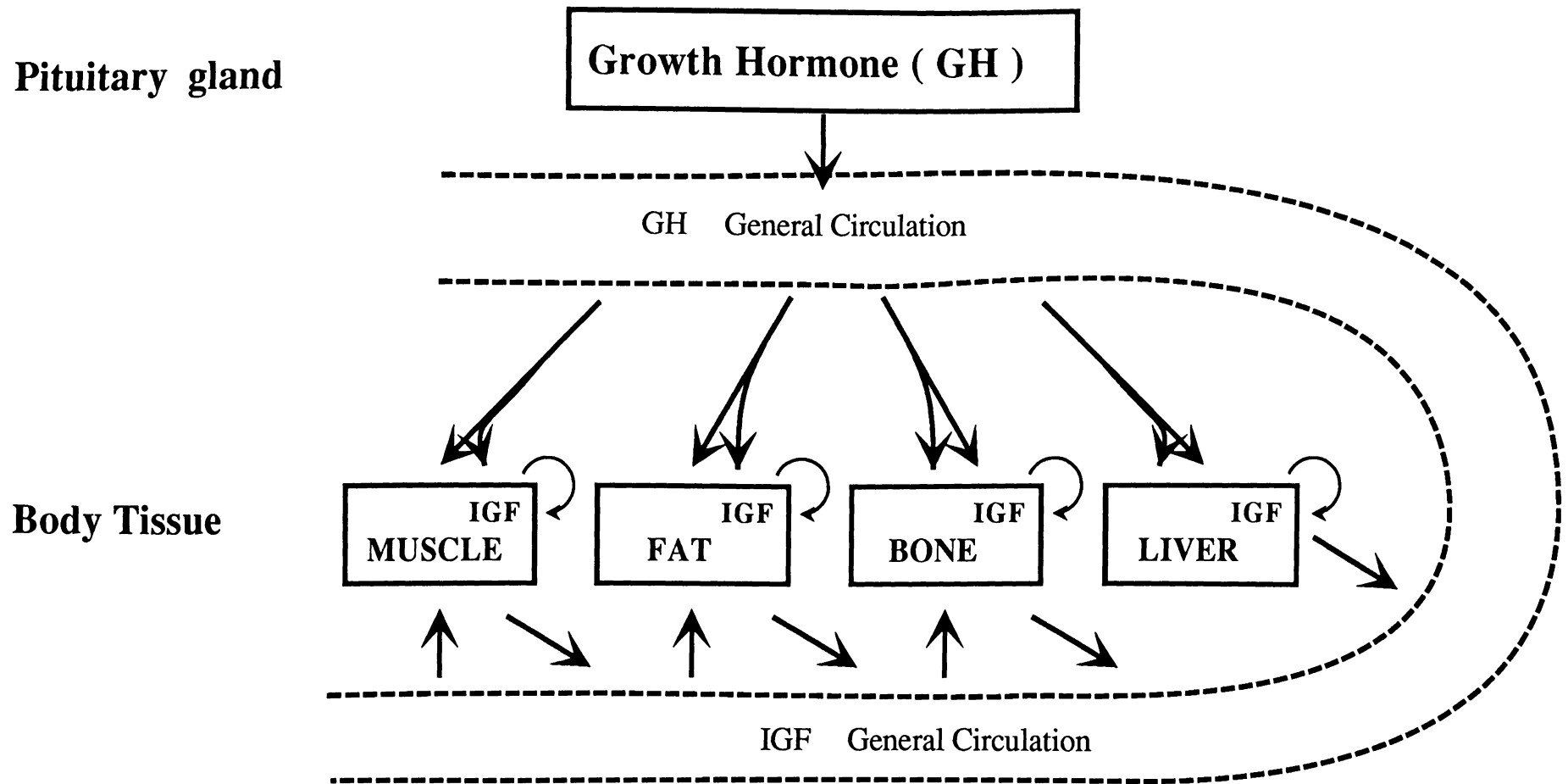


Figure 1.1 The GH - IGF Axis. Possible routes of GH action on growth of peripheral tissues.

(Modified from Bass & Clark, 1989)

GROWTH HORMONE

BIOCHEMISTRY AND SECRETION

Structure and Species Homology of GH

Based on common biological, immunological and structural features, GH is grouped with prolactin, placental lactogen (Nicoll, Mayer and Russell, 1986) and more recently, proliferin (Wallis, 1988). GH and prolactin probably arose from one ancestral molecule from which GH has shown less divergence than prolactin (Farmer and Papkoff, 1979). Plasma concentrations also show an evolutionary trend, levels of GH being relatively higher than prolactin in mammals, comparable to prolactin levels in reptiles and birds and relatively less than prolactin concentrations in amphibians (Nicoll and Licht, 1971).

Bovine GH (bGH) is a polypeptide chain of approximately 22 kDa in molecular weight and comprised of 191 amino acid residues (Havel, Kauffman and Elzinga, 1988). To attain its tertiary conformation the peptide folds and forms two disulphide bridges between cysteine residues. The sequence of residues shows heterogeneity, with the presence or absence of the n-terminal alanine and a residue substitution at position 127 (Wallis, 1973). Wallis (1973) compared the sequences of ovine GH (oGH) and bGH, finding differences at positions 99 and 130. Primate GH differs markedly in amino acid sequence from other mammalian GHs (Wallis, 1988; Hammerman, 1989), with a 35% to 40% difference in residue composition between bGH and human GH (hGH) suggesting that molecular evolution has been more divergent than the structural conformation of the molecules suggests (Wallis, 1973). Lack of homology between primate and non-primate GH has been isolated to two regions of the peptide, positions 42 to 49 and 184 to 191 (Rivero, Cascone and Biscoglio de Jimenez Bonino, 1990).

Complexity in the protein characteristics of GH led to the realisation that numerous post-translational changes to the molecule could take place (Miller and Eberhardt, 1983; Lewis, 1984). Lewis (1984) has reviewed these and extended earlier suggestions by

others to propose a hypothesis that GH is a pro-hormone which may have the most profound effects on somatic growth only after fragmentation. Certainly, distinct metabolic actions are associated with defined peptide sequences of GH (Liberti and Durham, 1983; Stevenson, Stebbing, Jones *et al.*, 1988), and *in vivo* production of various GH variants has been demonstrated (Markoff, Lee, Culler *et al.*, 1986; Caufriez, Frankenne, Englert *et al.*, 1990).

Plasma Concentrations of GH

Secretory Source of GH ~ GH is secreted from somatotropes in the anterior pituitary gland (Millard, 1989) in response to a variety of physiological and environmental cues. The pituitary gland is the primary, if not sole, site of GH synthesis as indicated by a decline of assayable GH in plasma to undetectable levels following hypophysectomy (Evans, Simpson, Marx *et al.*, 1943). In sheep, a reduction in skeletal growth rate then follows which is reversible with GH therapy (Young, Mesiano, Hintz *et al.*, 1989). Likewise, GH deficient children show increased height gains following treatment with GH or growth hormone releasing hormone (GRH) (Ross, Rodda, Tsagarakis *et al.*, 1987).

Patterns of GH Secretion ~ Many species show pulsatile secretion of GH. The rhythm of secretion ranges from well defined and regular in rats (Even, Danguir, Nicolaidis *et al.*, 1987) and dogs (French, Vaitkus, Cukerman *et al.*, 1987), to less synchronous in humans (Ho, Veldhuis, Johnson *et al.*, 1988) and apparently asynchronous in cattle (Gluckman, Breier and Davis, 1987) and sheep (Driver and Forbes, 1981). In ruminants, GH pulsatility is reportedly established *in utero* (Bassett and Gluckman, 1986; Reynolds, Ferrell, Robertson *et al.*, 1990). It has been noted that GH pulsatility limits the value of studies where assessment of GH release has been based on infrequent or limited sampling (Davis, Ohlson, Klindt *et al.*, 1977a; Gluckman *et al.*, 1987). In rats, pulse frequency is an important stimulus of growth. Hypophysectomised rats which received GH therapy as several intravenous boluses a day showed greater

weight gains than those which received the same daily dosage of GH as a continuous infusion (Clark, Jansson, Isaksson *et al.*, 1985). In other hypophysectomy models, the effectiveness of pulsatile GH therapy may be related to the extent of endogenous pulsatility in the species. For example, hypophysectomised lambs show a complete recovery of skeletal growth during three weeks of GH treatment at an injection frequency of three times a week (Young *et al.*, 1989).

Humans and other primates show a diurnal pattern of GH secretion (Quabbe, Schilling and Helge, 1966; Parker, Rossman and Vanderlaan, 1972; Kaler, Gliessman, Craven *et al.*, 1986). GH is elevated at night and during periods of deep sleep. There is some evidence that this capacity may be mediated by cholinergic mechanisms (Root, 1980) and lost with advancing age (Kaler *et al.*, 1986; Vermeulen, 1987). A diurnal variation in somatotrope sensitivity to GRH stimulation has also been documented (Schriock, Hulse, Harris *et al.*, 1987). There is no apparent diurnal variation of GH in cattle (Blom, Halse and Hove, 1976), goats (Tindal, Knaggs, Hart *et al.*, 1978) or sheep (Davis *et al.*, 1977a; Driver and Forbes, 1981; McMillen, Thorburn and Walker, 1987). Pregnant ewes may be an exceptional case as McMillen *et al.* (1987) clearly demonstrated a sinusoidal diurnal rhythm of GH with elevated concentrations at night. Using the same sampling regime they were not able to detect any such rhythm in the foetuses during late gestation.

Distinct changes in GH secretion occur with the ontogenetic development of the foetus through to adulthood. In the ovine foetus, plasma GH increases from 100 to 139 days of gestation, falls as the animal approaches term and then declines rapidly immediately following birth (Bassett and Gluckman, 1986). Similar changes have been reported for bovine foetuses (Reynolds *et al.*, 1990). The perinatal drop in GH has been determined to be predominantly due to decreased secretion from the pituitary gland rather than increased clearance of GH from plasma (Power, Ball and Gluckman, 1988). In humans, there is an association of elevated GH concentrations with premature infants (Samaan,

Schultz, Johnston *et al.*, 1987) which may be a reflection of the large changes in plasma concentrations of GH which take place at birth.

Increased growth takes place during puberty, requiring contributions from both the sexual steroids and GH (Brook, Hindmarsh and Stanhope, 1988). In pubertal boys, increased GH is postulated to result from changes in pulse amplitude rather than frequency (Mauras, Blizzard, Link *et al.*, 1987). While studies on ruminants have not been performed to document precisely the changes in GH which take place during puberty, at this time in the rat the adult patterns of GH secretion develop (Jansson, Edén and Isaksson, 1985). Differences in GH with sexual status have been reported in ruminants; ram lambs have greater concentrations of GH in plasma than wethers (Davis *et al.*, 1977a). However administration of testosterone to wethers does not have an immediate effect on plasma GH (Lobley, Connell, Buchan *et al.*, 1987). In contrast, anabolic or oestrogenic steroid treatment of steers does increase GH concentrations in plasma (Gopinath and Kitts, 1984; Breier, Gluckman and Bass, 1988b).

The progression of pregnancy in ewes is associated with a threefold increase in GH concentrations as parturition approaches (Blom, Hove and Nedkvitne, 1976) although this is suggested to be linked with nutritional status (Blom *et al.*, 1976; Gluckman *et al.*, 1987). Throughout gestation, GH concentrations of ruminant fetuses still remain several-fold higher than those of the dam (McMillen *et al.*, 1987; Reynolds *et al.*, 1990). Finally, there is a reported decline of plasma GH with old age in humans which is expressed as a reduction in the amplitude of GH peaks without a change in baseline concentrations (Vermeulen, 1987).

GH Binding Protein ~ In plasma, GH circulates complexed to a specific binding protein which has the same amino acid sequence as the extracellular region of the membrane GH receptor (Leung, Spencer, Cachianes *et al.*, 1987). The apparent molecular weight of the binding protein is 51 kDa, approximately 40% of the isolated

receptor (Spencer, Hammonds, Henzel *et al.*, 1988). It has been estimated that 50% of hGH in plasma is complexed by the binding protein, this proportion declining as GH concentrations increase (Baumann, Amburn and Shaw, 1988). An ontogenetic variation of the binding protein in human plasma has been demonstrated whereby levels peak during early adulthood (Daughaday, Trivedi and Andrews, 1987), but diurnal and sexual differences are not apparent (Snow, Shaw, Winer *et al.*, 1990).

Hormonal Control of GH Secretion

Endogenous GH secretion is primarily controlled by two hypothalamic hormones; GRH which stimulates GH secretion and somatostatin (SRIF) which inhibits GH release (Brook *et al.*, 1988; Millard, 1989). Hypothalamic-pituitary disconnection in sheep does not reduce GH mRNA and it was suggested that this was due to the concurrent removal of both the major stimulatory and inhibitory influences (Hamernik, Crowder, Nilson *et al.*, 1986). Modulating effects of other pituitary stimulants on GH secretion are also apparent. Of particular interest are the effects of thyrotropin releasing hormone (TRH), IGF1 and feedback control by GH itself. The major hormones which influence GH secretion are discussed below.

Growth Hormone Releasing Hormone ~ GRH is a peptide of 44 amino acids which, in numerous truncated forms down to GRH1-21, can stimulate GH secretion from the anterior pituitary gland to the same extent as the entire peptide (Davies and Johnston, 1987). In addition to hypothalamic GRH, contributions to plasma levels are probably made from the gastrointestinal tract (Shibasaki, Kiyosawa, Masuda *et al.*, 1984). GRH is secreted in a pulsatile manner in humans (Vance, 1990) and is likely to affect GH release via stimulation of cyclic AMP in somatotropes (Ray and Wallis, 1988). Plasma levels of GRH may be regulated by diet (Sopwith, Penny, Lytras *et al.*, 1987) and reduce with fasting (Penny, Sopwith, Patience *et al.*, 1986). In rats, hypothalamic GRH is detectable in the late-gestational foetus and increases after birth (Jansson, Ishikawa, Katakami *et al.*, 1987). There is no sexual dimorphism in GRH content of the hypothalamus (Jansson *et*

al., 1987) and no apparent decline in the effectiveness of GRH as a GH stimulant with aging in humans (Pavlov, Harman, Merriam *et al.*, 1986). Heterologous GRHs are apparently less potent stimuli of GH from rat pituitary culture than is the homologous GRH (Baird, Wehrenberg and Ling, 1986).

Treatment *in vivo* with exogenous GRH has resulted in immediate, significant elevation of plasma GH in several agricultural species; cattle (Moseley, Krabill, Friedman *et al.*, 1984; Moseley, Huisman and Van Weerden, 1987), sheep (Della-Fera, Buonomo and Baile, 1986a; Wrutniak, Cabello, Charrier *et al.*, 1987), pigs (Della-Fera, Buonomo and Baile, 1986b; Petitclerc, Pelletier, Lapierre *et al.*, 1987) and chickens (Taylor, Ball and Leung, 1986). The application of GRH therapy to correct some forms of GH deficiency (Borges, Blizzard, Evans *et al.*, 1984; Low, Wang, Cheung *et al.*, 1988) and investigate the hormonal mechanism of acromegaly (Chiodini, Liuzzi, Dallabonzana *et al.*, 1985; Smals, Pieters, Smals *et al.*, 1988) is also being extensively researched in humans.

In sheep, the GH response to GRH administration by intravenous or subcutaneous injection is transient and dose dependent (Della-Fera *et al.*, 1986b), becoming refractory with repeated administration (Della-Fera *et al.*, 1986a). GRH treatment by infusion also does not permanently elevate plasma GH in humans (Losa, Bock, Schopohl *et al.*, 1984) or rats (Wehrenberg, Brazeau, Ling *et al.*, 1984). This phenomenon is considered to be a consequence of somatotrope desensitization (Vance, Kaiser, Rivier *et al.*, 1986; Rittmaster, Loriaux and Merriam, 1987) or depletion of intracellular GH (Davis, Sheppard, Shakespear *et al.*, 1986; Richardson and Twente, 1988). Several studies *in vitro* have investigated the regulation of GH secretion by GRH from ruminant pituitary cultures. The GH response is dose dependent (Glenn, 1986; Blanchard, Goodyer, Charrier *et al.*, 1987) and preparations from neonates are more sensitive than foetal cells (Silverman, Bettendorf, Kaplan *et al.*, 1989). The GH response to GRH is dependent on Ca^{2+} status (Barinaga, Bilezikjian, Vale *et al.*, 1985; Hart, Ray and Wallis, 1988) and is

mediated both by GH release and stimulation of GH gene transcription (Barinaga *et al.*, 1985).

Somatostatin ~ SRIF is a peptide of 14 amino acids which arises as a result of cleavage from a 92 amino acid pro-hormone. Two truncated peptides show biological activity, SRIF1-14 and SRIF1-28 (Millard, 1989). SRIF was extracted and characterised from the ovine hypothalamus (Brazeau, Vale, Burgus *et al.*, 1973) but is also located in other tissues including, like GRH, other regions of the central nervous system (Terry and Crowley, 1986) and the gastrointestinal tract (Patel, Weir and Reichlin, 1975).

A variety of studies performed *in vitro* (Yokoya and Friesen, 1986; Blanchard *et al.*, 1987) and *in vivo* (Davis, 1975; Taylor *et al.*, 1986) demonstrate that SRIF depresses pituitary secretion of GH. Differences in the potency of SRIF to depress GH secretion have been associated with age (Bowers, Reynolds, Chang *et al.*, 1981; Silverman *et al.*, 1989) and the GH-releasing factor which was used as a GH stimulant (Taylor *et al.*, 1986). There is also differential sensitivity of basal and stimulated GH secretion as to the effectiveness of SRIF as an inhibiting agent (Davis, 1975; Terry and Crowley, 1986; Padmanabhan, Enright, Zinn *et al.*, 1987). Pituitary cells in culture show a capacity for SRIF to depress GH synthesis and secretion in the absence of a stimulatory releasing factor (Yokoya and Friesen, 1986; Rittmaster *et al.*, 1987).

SRIF shows a moderate decline in portal plasma concentrations during GRH secretion (Plotsky and Vale, 1985). It has been postulated that each peptide undergoes a rhythmic surge in secretion out of phase with the other, which accounts for the troughs (SRIF peak) and peaks (GRH peak) of plasma GH (Tannenbaum and Ling, 1984). The suggestion has also been made that SRIF regulates the timing of GH peaks while GRH regulates the peak magnitude (Kraicer, Cowan, Sheppard *et al.*, 1986). Co-incubation of pituitary cells with GRH and SRIF reduces the extent of GH depletion and attenuates the

GH response to GRH (Clayton and Bailey, 1987), but at equal concentrations GRH stimulation prevails over SRIF inhibition (Blanchard *et al.*, 1987).

Thyrotropin Releasing Hormone ~ TRH, a physiological stimulant of the release of thyroid stimulating hormone from the pituitary gland, has also been implicated as a modulator of GH secretion (Harvey, 1990). The relative potencies of GRH and TRH as independent stimulants of GH varies between species and experimental conditions. Taylor *et al.* (1986) have concluded that in chickens TRH is more potent than GRH *in vivo*, but GRH is more potent than TRH *in vitro*. Cattle studies *in vivo* indicate that TRH is a poorer stimulant of GH than GRH (Coxam, DaVicco, Opmeer *et al.*, 1987; Lapierre, Petitclerc, Pelletier *et al.*, 1987) and this is supported by *in vitro* data (Ingram and Bicknell, 1986). In sheep, a GH response to TRH appears to be dependent on age. No GH response was elicited with TRH treatment in lambs of two to twelve weeks old (Wrutniak *et al.*, 1987) although Johnsson (1984) established that GH sensitivity to TRH was present predominantly between six and 26 weeks of age. Using pituitary culture, GRH is a considerably more potent stimulus of GH from ovine prepubertal cells than TRH (Blanchard *et al.*, 1987), although the capacity of the pituitary to secrete GH in response to TRH is present in cells from adult sheep (Takahara, Arimura and Schally, 1974).

Whether or not TRH is an independent stimulant of GH secretion, there is a capacity for a synergistic interaction with GRH to stimulate GH secretion. This is clearly evident in cattle (Brazeau, Lapierre, Petitclerc *et al.*, 1986; Lapierre *et al.*, 1987). In sheep, only one study has been performed *in vivo* to investigate whether GRH and TRH act synergistically to stimulate GH (Wrutniak *et al.*, 1987); the effect of TRH was to delay the GH response to GRH. A study with ovine pituitary cells *in vitro* has determined that the effect of TRH on GRH-stimulated secretion of GH may be inhibitory or stimulatory depending on the Ca^{2+} concentration in the incubation medium (Hart *et al.*, 1988).

GH Feedback ~ Evidence has accumulated which suggests that a feedback control of GH secretion by plasma GH and/or IGF1 exists. In rats with elevated plasma GH and IGF1, pituitary content of GH mRNA is reduced (Yamashita, Slanina, Kado *et al.*, 1986). GH treatment of normal rats also reduces the amplitude of endogenous GH peaks, as does IGF1 administration (Abe, Molitch, Van Wyk *et al.*, 1983). Demonstrating more clearly a direct effect, GH treatment of humans depressed the GH response to GRH without altering plasma IGF1 concentrations (Ross, Borges, Grossman *et al.*, 1987). A similar response was observed after a more prolonged period of GH treatment during which plasma IGF1 concentrations did increase (Rosenthal, Hulse, Kaplan *et al.*, 1986). Bovine pituitary cells *in vitro* show a suppression of basal but not stimulated GH release following treatment with GH (Glenn, 1986) suggesting that, as for SRIF inhibition, GRH may override the feedback control of GH on the somatotropes.

IGF1 Feedback ~ Studies *in vitro* support the concept of a direct effect of IGF1 on GH secretion. IGF1 down-regulates GH mRNA in a carcinoma cell line (Yamashita, Ong and Melmed, 1987) and depresses basal and stimulated GH secretion in rat pituitary cells (Berelowitz, Szabo, Frohman *et al.*, 1981; Yamashita and Melmed, 1986). It has been postulated that feedback control by IGF1 operates initially by stimulating hypothalamic SRIF release, then by direct effects on the pituitary (Berelowitz *et al.*, 1981), possibly affecting different populations of somatotropes than those affected by SRIF (Hoeffler, Hicks and Frawley, 1987). In contrast to the effects obtained with rat pituitary cells in culture, GH release from bovine pituitary cells was not suppressed by IGF1 (Glenn, 1986). Intracerebroventricular administration of IGF1 to sheep was also ineffective in reducing plasma concentrations of GH (Spencer, Bass, Hodgkinson *et al.*, 1991) although a similar treatment of foetal pigs resulted in lower plasma levels of GH for several hours following injection (Spencer, Macdonald, Buttle *et al.*, 1991). These studies suggest that there may be species variation as to whether IGF1 participates in the feedback control of GH secretion from the pituitary.

Effect of Nutrition On GH in Ruminants

In sheep and cattle, plasma concentrations of GH are increased by dietary restriction (Bassett, Weston and Hogan, 1971; Kung, Huber, Bergen *et al.*, 1984) and fasting (Driver and Forbes, 1981; Moseley, Alaniz, Claflin *et al.*, 1988). Characteristics of the GH response include an increase in peak frequency (Bass, Davis, Peterson *et al.*, 1984), peak amplitude (Breier *et al.*, 1988b; Moseley *et al.*, 1988; Thomas, Mercer, Karalis *et al.*, 1990) or both (Driver and Forbes, 1981). Sheep predominantly exhibit changes in pulse frequency (Driver and Forbes, 1981; Bass *et al.*, 1984) while cattle show a major change in pulse amplitude (Breier *et al.*, 1988b; Moseley *et al.*, 1988).

Restricted feeding also increases the ovine pituitary content of GH mRNA but not the content of GH itself (Thomas *et al.*, 1990). This suggests that the elevated plasma concentrations of GH are a result of increased GH secretion, although the half-life for plasma GH in fasted lambs is also longer than in fed lambs (Trenkle, 1976). Another feature of pituitary responsiveness which is affected by nutrition is the effectiveness of GRH as a GH stimulant. Animals on a restricted diet show a more persistent elevation of GH in response to GRH than those fed *ad libitum* (Hart, Chadwick, Coert *et al.*, 1985) and the number of animals responding to a GRH challenge and peak amplitude of the GH response are both greater before than after feeding (Moseley *et al.*, 1988).

Dietary components which are associated with changes in GH concentrations include protein (Kung *et al.*, 1984; Waghorn, 1986) energy (Waghorn, 1986) and trace elements (Emery, Luoma, Liesman *et al.*, 1986). A neural mechanism is also implicated. Rumen distension in goats decreases plasma GH (Tindal, Blake, Simmonds *et al.*, 1985), and Driver and Forbes (1981) postulated that a neural reflex was operational in reducing plasma GH as sheep were feeding.

BIOLOGICAL ACTION

Growth in post-natal sheep is impaired following hypophysectomy and restored with GH therapy (Young *et al.*, 1989), thus demonstrating a dependence on GH in the absence of nutritional constraint. Studies to manipulate normal growth have been undertaken in numerous species using putative growth hormones and various antisera generated against growth-promoting hormones. In an early trial, GH administration to cattle increased liveweight gain and bone length (Brumby, 1959) but experiments since this have not conclusively supported an effect of exogenous GH on weight gain (Peters, 1986; McShane, Schillo, Boling *et al.*, 1989). Measurements of bone growth also showed variation in their response to GH treatment (McShane *et al.*, 1989). A more dramatic and repeatable consequence of GH treatment is a reduction in carcass fatness (Peters, 1986; McShane *et al.*, 1989).

Results have been similar in studies of GH administration to sheep. Increased weight gains have been demonstrated in some studies (Johnsson, Hart and Butler-Hogg, 1985; Bass, Hodgkinson, Breier *et al.*, 1991a) but not others (Muir, Wien, Duquette *et al.*, 1983; Rosemberg, Thonney and Butler, 1989). Bone length is apparently unaffected (Rosemberg *et al.*, 1989) but bone content as a proportion of the carcass increases (Johnsson *et al.*, 1985; Butler-Hogg and Johnsson, 1987). Associated with the changes in bone proportions is an increase in muscle (Johnsson *et al.*, 1985; Rosemberg *et al.*, 1989) and a reduction in carcass fat (Wagner and Veenhuizen, 1978; Muir *et al.*, 1983). Chemical analyses of composition have also revealed increased protein (Rosemberg *et al.*, 1989) and possibly water content (Muir *et al.*, 1983) of the carcass.

The development of immunization techniques to potentiate or inhibit the action of hormones and receptors in the growth axis is an alternative approach to the administration of exogenous compounds to manipulate growth. There have been recent reports of the actions of GH *in vivo* being enhanced by specific GH antibodies. GH stimulated milk production in ewes has been further increased (Pell, Johnsson, Pullar *et al.*, 1989) as has

GH stimulated weight gain in dwarf mice (Bomford and Aston, 1990). An antiidiotypic antiserum to GH antibodies has also been developed which competes with radiolabelled GH for binding to liver membranes and stimulates weight gain in hypophysectomised rats (Gardner, Morrison, Stevenson *et al.*, 1990).

Manipulation of growth by active immunization against the primary inhibitory hormone, somatostatin (SRIF), has also been investigated in sheep. Spencer, Garssen and Hart (1983) immunised young lambs against SRIF and observed an increase in weight and height gain. A sexual difference in responsiveness was noted, with females more sensitive to immunization than males (Spencer, Hallett and Fadlalla, 1985). In older lambs, immunization against SRIF did not affect subsequent growth, although pituitary glands were marginally enlarged (Bass, Gluckman, Fairclough *et al.*, 1987). Both the latter studies used castrated males suggesting, together with the earlier observation (Spencer *et al.*, 1985), that sexual status and age may be important determinants of responsiveness to immunization against SRIF.

MECHANISM OF ACTION

An evaluation of the mechanism of action of GH has been complicated by studies in which heterologous GH has been used to investigate growth restoration in growth-deficient models, by uncertainty about the chemical nature of the GH product (Boyd and Bauman, 1989) and the extensive use of *in vitro* systems as a research tool (Florini, 1987). Some general effects of GH are consistently observed though, and together the published reports broadly classify GH effects as metabolic or mitogenic (Boyd and Bauman, 1989). It must also be considered that at least some GH effects are mediated by stimulation of local IGF production in the target tissue, thus being indirect (Wallis, 1988; Isgaard, Nilsson, Vikman *et al.*, 1989).

Action of GH on Skeletal Muscle

Administration of GH to cattle causes nitrogen uptake (Moseley, Krabill and Olsen, 1982; Eisemann, Hammond, Bauman *et al.*, 1986), and in sheep the protein gain with GH treatment is largely due to increased synthesis of both collagen and non-collagen components of muscle (Pell and Bates, 1987). The response of different muscles to GH is not consistent. Greater effects of GH on protein synthesis were noted for red than white muscles (Pell and Bates, 1987), and in hypophysectomised rats the transition of Type 1 to Type 2 muscle fibres is influenced by GH therapy (Ayling, Moreland, Zanelli *et al.*, 1989).

There is evidence that the anabolic effects of GH may not be direct. Pell and Bates (1989) demonstrated comparable protein synthesis with GH and IGF1 treatment of dwarf rats. The evidence *in vitro* that GH has direct metabolic effects on muscle cells is limited (Ewton and Florini, 1980), but the specialised characteristics of cultured cells have to be considered in this evaluation (Florini, 1987). In satellite cells from foetal lambs (Harper, Soar and Buttery, 1987) and adult sheep (Roe, Harper and Buttery, 1989), no effect of GH on protein metabolism has been observed.

Mitogenic effects of GH on muscle have been reported, although in sheep there is little alteration of fibre number, fascicle number and connective tissue content after birth (Sivachelvan and Davies, 1986). DNA synthesis shows a dependence on GH, as demonstrated in the hypophysectomised rat (Kostyo and Nutting, 1973; Goldspink and Goldberg, 1975), although incubation *in vitro* of muscle from hypophysectomised rats with GH failed to increase DNA synthesis (Goldspink and Goldberg, 1975). Administration of GH to normal rats during the post weaning phase of growth also does not alter myonuclei and satellite cell populations (Beerman, Liboff, Wilson *et al.*, 1983). A direct mitogenic effect of GH to differentiate muscle cells in culture has been demonstrated but this phenomenon appears to be restricted to particular cell lines (Nixon and Green, 1984) and is not apparent in bovine (Gospodarowicz, Wesemen, Moran *et*

al., 1976) or rat myoblasts (Ewton and Florini, 1981). While GH may directly differentiate muscle cells, possibly during embryonic or foetal development, the capacity to stimulate hyperplasia during later development appears limited. Changes in DNA synthesis with GH-regulated muscle growth may be essentially hypertrophic in nature and possibly mediated by IGFs.

Action of GH on Adipose Tissue

A characteristic consequence of GH administration to sheep and cattle is a reduction in carcass fat and elevation of plasma concentrations of free fatty acids (Peters, 1986; Bass *et al.*, 1991a). Observations such as these have resulted in a massive research effort into elucidating the mechanisms by which GH affects adipose tissue. Recently, this has been reviewed by Davidson (1987) and, with particular reference to ruminants, by Vernon and Flint (1989). Both reviews considered the complicated nature of GH effects on adipose tissue and attempt to reconcile the numerous conflicting observations.

The effects of GH *in vitro* on adipose tissue from hypophysectomised rats were initially noted as an increase in glucose utilisation and conversion to lipid (insulin-like) followed by reduced glucose utilisation, lipolysis and a refractory state to further insulin-like stimulation by GH (anti-insulin-like) (Goodman, 1981). The insulin-like effect of GH on adipose tissue from normal rats could only be elicited following a period of pre-incubation *in vitro* (Herington, Stevenson and Andrew, 1985). During this time the return of responsiveness was associated with increased availability of GH binding sites (Herington *et al.*, 1985) but comparable GH binding in responsive and refractory tissue has also been reported, suggesting post-receptor refractoriness (Grichting, Levy and Goodman, 1983). There is evidence that the insulin-like effect of GH is an inherent property of the hormone rather than a characteristic of the adipocyte (Cameron, Kostyo, Adamafio *et al.*, 1988), although the physiological significance of this effect is questionable owing to its appearance only in GH deficient states (Davidson, 1987; Vernon and Flint, 1989).

The secondary anti-insulin-like effect of GH requires prolonged exposure to GH and it appears to be this effect which is reflected in reduced carcass fatness with GH treatment. In their consideration of recent studies testing whether GH stimulates lipolysis directly, Vernon and Flint (1989) concluded that the supporting evidence was questionable although GH was likely to enhance lipolysis stimulated by other physiological compounds, as demonstrated by Peters (1986).

Independent of the insulin-like and anti-insulin-like effects of GH, is antagonism of insulin stimulated lipogenesis (Davidson, 1987). This has been demonstrated *in vitro* in adipose tissue from sheep (Vernon, 1982) and cattle (Etherton, Evock and Kensinger, 1987). Prolonged exposure to GH is required and GH alone does not affect fatty acid synthesis, but insulin stimulated synthesis is reduced in the presence of GH (Vernon, 1982; Etherton *et al.*, 1987).

Finally, the evidence of a mitogenic action of GH on adipose tissue *in vivo* is limited (Vernon and Flint, 1989) although differentiation of pre-adipocytes into adipocytes with GH incubation has been demonstrated with cell culture (Nixon and Green, 1984; Zezulak and Green, 1986). The differentiating capacity of GH appears to be direct as IGF1 substitution is ineffective (Nixon and Green, 1984).

Action of GH on Bone

Whether or not particular bone measurements in sheep alter with growth depends on the site from which the measurements are taken (Searle, Graham and Donnelly, 1989), but the dependence of bone growth on GH has been demonstrated in hypophysectomised sheep (Young *et al.*, 1989) and normal cattle (McShane *et al.*, 1989) receiving GH treatment. The mechanisms controlling GH effects on bone growth have been extensively studied in the rat. Isaksson and co-workers have demonstrated that GH has both direct and indirect effects on the epiphyseal growth plate (Isaksson *et al.*, 1987).

Local administration of GH and IGF1 to the proximal tibia of hypophysectomised rats stimulates bone growth, although GH is the more potent stimulus (Isgaard *et al.*, 1986). GH also regulates IGF1 mRNA in cultured chondrocytes (Nilsson, Carlsson, Isgaard *et al.*, 1990), supporting the idea of a role for locally produced IGF1 in bone growth. The effects of GH and IGF1 on chondrocyte colonisation in culture differ (Lindahl, Isgaard, Carlsson *et al.*, 1987), suggesting that their roles in chondrocyte development may also differ. These observations support the theory that GH matures undifferentiated chondrocytes into IGF1-sensitive and IGF1-secreting cells. Paracrine or autocrine effects of IGF1, secreted from the differentiated chondroblasts following stimulation by GH, then regulate clonal expansion into fully mature cells in the hypertrophic zone of the epiphyseal growth plate (Isaksson *et al.*, 1987). This theory is similar to the dual effector theory developed to explain the general mitogenic actions of GH (Green *et al.*, 1985).

INSULIN-LIKE GROWTH FACTORS

BIOCHEMISTRY AND SECRETION

Structure and Species Homology of the IGFs

Based on structural and functional homologies, IGF1 and IGF2 together with relaxin and insulin are grouped together as the insulin peptide family (Steele and Elsasser, 1989). Human IGF1 (hIGF1) is a basic, single chain polypeptide of 70 amino acids and a molecular weight of 7649. Three disulphide bridges assist in maintaining the conformation of the molecule. The overall sequence homology with pro-insulin is approximately 50%, but in the A and B regions of the molecules sequence homology is greater, at 60% to 70% (Rinderknecht and Humbel, 1978a). Human IGF2 (hIGF2) is a single chain polypeptide of 67 amino acids, slightly acidic in nature and with a molecular weight of 7471. Three disulphide bridges are also present in the IGF2 molecule and the homology of amino acids with IGF1 is 62%. Shared homology between IGF1, IGF2 and pro-insulin approximates 50% (Rinderknecht and Humbel, 1978b). IGF1 shows

43% and IGF2 41% amino acid homology with the A and B regions of insulin (Zangger, Zapf and Froesch, 1987).

Unlike insulin and relaxin which are double-stranded molecules, the IGFs are single stranded, more like the pro-hormones from which relaxin and insulin are cleaved (Steele and Elsasser, 1989). The conformation of insulin and the IGFs is similar though (Blundell, Bedarkar, Rinderknecht *et al.*, 1978), which is important in the context of their biological actions. A single cleavage in the C region of IGF1 and IGF2, resulting in a double-stranded molecule, differentially affects binding to receptors but has little effect on the complexing of IGFs to binding proteins (Jansen, van Buuls-Offers, Hoogerbrugge *et al.*, 1990), indicating that the functional binding sites on IGFs differ in location for the receptor and binding proteins (Froesch, Schmid, Schwander *et al.*, 1985).

Bovine IGF1 (bIGF1) is identical to hIGF1 and bovine IGF2 (bIGF2) differs by three amino acids, all in the C region, from hIGF2 (Honegger and Humbel, 1986). Species variation in amino acid composition of the IGFs has been noted to occur predominantly in the C region of the molecules (Humbel, 1984) and is greater for IGF2 than for IGF1 (Zangger *et al.*, 1987). The sequence of ovine IGF1 (oIGF1) differs from the human and bovine sequences by only one residue, and ovine IGF2 (oIGF2) differs from bIGF2 by a single residue also (Francis, McNeil, Wallace *et al.*, 1989). There appears to be no difference between foetal and adult sequences of the oIGFs (Francis *et al.*, 1989; Hey, Browne, Simpson *et al.*, 1989).

Plasma Concentrations of IGFs

Secretory Sources of IGFs ~ Difficulties in determining the secretory source of IGFs are due to there being no tissue containing greater concentrations than are measured in plasma (Froesch *et al.*, 1985). Unlike most polypeptide hormones, which are stored intracellularly and quickly released in response to appropriate stimuli, IGF synthesis and secretion is apparently rapid and continuous (Schwander *et al.*, 1983; Lund, Han,

D'Ercole *et al.*, 1987). A reflection of this mode of secretion is the stable plasma concentrations. Plasma IGF1, while proposed to be primarily hepatic in origin (Schwander *et al.*, 1983), may be from a number of tissues which express IGF1 mRNA (Murphy, Bell and Friesen, 1987; Han, Lund, Lee *et al.*, 1988). Tissue localisation of IGF2 mRNA is also extensive (Brown, Graham, Nissley *et al.*, 1986; Lund, Moats-Staats, Hynes *et al.*, 1986), but greatest in the brain (Murphy *et al.*, 1987). The cellular localisation of IGF mRNA in foetal tissue is predominantly to connective tissue and cells of mesenchymal origin (Han, D'Ercole and Lund, 1987), and mRNA transcripts of several sizes are reported for both IGFs (Brown *et al.*, 1986; Lund *et al.*, 1986; Rotwein, 1986).

To further support the opinion that there are multiple secretory tissues throughout the body, and considering the caution that expression of mRNA does not necessarily indicate synthesis of the protein product (Sara and Hall, 1990), several studies have demonstrated immunodetection of IGFs in various tissues (Andersson, Billig, Fryklund *et al.*, 1986; Hansson, Jennische and Skottner, 1987; Bautista, Mohan and Baylink, 1990), and co-localisation of the IGFs and mRNA by immunological and hybridization chemistry (Bortz, Rotwein, DeVol *et al.*, 1988; Romanus *et al.*, 1988). The presence of IGF1 and IGF2 has also been reported in a number of body fluids other than plasma *e.g.* saliva (Costigan, Guyda and Posner, 1988), colostrum (Francis, Upton, Ballard *et al.*, 1988) and lymph (Binoux and Hossenlopp, 1988).

Patterns of IGF Secretion ~ Large changes in mRNA expression, tissue concentrations and plasma levels of IGFs take place during ontogenesis (Breier, Gluckman and Bass, 1988c; Sara and Hall, 1990). The level of IGF2 mRNA is higher in foetal than in adult tissue from rats (Lund *et al.*, 1986) and greater than that of IGF1 mRNA in the foetus (Han *et al.*, 1988). The patterns of mRNA expression throughout foetal development also differ between the IGFs. IGF2 mRNA is relatively constant while IGF1 mRNA rises several-fold during mid-gestation (Rotwein, Pollock, Watson *et*

al., 1987). After birth, IGF2 mRNA remains high in the neonatal rat, particularly in liver, skeletal muscle and the central nervous system (Brown *et al.*, 1986), then declines initially in the liver and skeletal muscle but remains present in the central nervous system throughout adult life (Beck, Samani, Byrne *et al.*, 1988). IGF1 mRNA increases post-natally to the greatest extent in liver. The ontogenetic timing of change differs from the hepatic decline in IGF2 mRNA suggesting that the IGFs are differentially controlled within a tissue (Beck *et al.*, 1988).

Reflecting mRNA levels, IGF2 concentrations in foetal plasma from rats are higher than in maternal plasma, declining after birth to adult levels (Moses, Nissley, Short *et al.*, 1980). In human plasma, IGF2 concentrations are low prenatally and increase after birth to concentrations greater than IGF1 in adult life (Enberg and Hall, 1984). Plasma concentrations of IGF2 are also greater than IGF1 concentrations in adult cattle (Davis, Gluckman, Hart *et al.*, 1987). Plasma concentrations of IGF1 show the reciprocal pattern of development to that of IGF2, being low in the foetus and neonate, and increasing after this time (Daughaday, Parker, Borowsky *et al.*, 1982). IGF1 shows a pubertal peak in concentrations which then stabilises at adult levels (Handelsman, Spaliveriero, Scott *et al.*, 1987; Hall, Lundin and Póvoa, 1988). There is evidence that the pubertal peak in IGF1 is regulated by changes in GH secretion and modulated by sexual steroids (Rappaport, Prevot and Brauner, 1987). In humans, plasma IGF1 may decline with advancing age (Vermeulen, 1987).

In sheep, changes in plasma IGFs during ontogenesis have been examined (Gluckman and Butler, 1983; Van Vliet, Styne, Kaplan *et al.*, 1983). Plasma IGF1 concentrations increase in the foetus during latter gestation and rise soon after birth. By one month of age the concentrations are similar to those of adults and significantly greater than foetal levels (Gluckman and Butler, 1983; Van Vliet *et al.*, 1983). Plasma concentrations of IGF2 in the ovine foetus are constant throughout pregnancy, and higher than in adults. A decline in concentrations immediately before birth is followed by little subsequent change

post-natally. These developmental patterns are similar to those reported for the rat, although the timing of changes in relation to birth differs (Moses *et al.*, 1980). Newborn calves show a distinct developmental pattern of IGF1 concentrations with a decline during the first few weeks of life, followed by an increase through the remaining pre-pubertal period (Breier *et al.*, 1988c). There is also evidence that IGF1 concentrations differ with sexual status in sheep, rams with greater concentrations in plasma than non-pregnant ewes. Little change in IGF1 occurs with pregnancy in ewes (Van Vliet *et al.*, 1983).

As highlighted by Hansson (1990) there is a characteristic time schedule for each tissue and organ with respect to their IGF expression. Characterisation of the species-specific developmental patterns is therefore important to elucidate optimal timing for the study of growth or manipulation through the GH-IGF axis.

IGF Binding Proteins ~ In plasma the IGFs are complexed to plasma binding proteins which are equally as extensive in their distribution of secretory sites (Clemmons, 1989; Hill, Clemmons, Wilson *et al.*, 1989). The binding proteins function to increase the half-life of plasma IGFs, possibly acting as a storage protein to ensure a constant supply of IGFs to target tissues (Baxter, 1986; Sara and Hall, 1990). There are three major molecular forms of binding protein, two showing similar affinity for IGF1 and IGF2 and the third with a preference for IGF2 (Clemmons, 1989; Martin, Willetts and Baxter, 1990). The binding proteins show species specificity in their affinity for binding the IGFs (Rechler, Zapf, Nissley *et al.*, 1980), and one shows a strong dependence on GH status in rats (Moses, Nissley, Passamani *et al.*, 1979) and humans (White, Nissley, Moses *et al.*, 1981). There is an ontogenetic change in the predominant form of binding protein in plasma (Donovan, Oh, Pham *et al.*, 1989), and secretion of binding proteins from cultured muscle cells also alters with the state of differentiation of the cells (McCusker and Clemmons, 1988).

Studies in sheep indicate that there are two sizes of binding protein (Butler and Gluckman, 1986; Bachrach, Liu, Burrow *et al.*, 1989), with IGF1 in adult plasma being predominantly complexed to the larger binding protein and IGF2 to the smaller protein (Butler and Gluckman, 1986). The small binding protein has since been further resolved into one molecule with similar affinity for both IGFs and one with a strong preference for IGF2 (Hodgkinson, Moore, Napier *et al.*, 1989a). The plasma half-life of IGF1 bound to the smaller binding protein is less than that of the larger binding protein complex and there is evidence that this is due to a transfer of IGF1 from the smaller to the larger binding protein complex (Davis, Hodgkinson, Moore *et al.*, 1989) and faster clearance from plasma. IGF1 which is not complexed to a binding protein is cleared very quickly from ovine plasma (Francis, McNamara, Filsell *et al.*, 1988). Secretion of the ovine binding proteins in culture (Bachrach *et al.*, 1989) and measurement in plasma (Hodgkinson, Davis, Burleigh *et al.*, 1987; Hodgkinson, Bass and Gluckman, 1991) suggest that the binding proteins are differentially regulated by hormonal stimuli and nutritional status.

It remains unknown as to whether the IGFs dissociate from binding proteins in target tissues or whether the hormone-binding protein complex interacts with the receptor (Froesch *et al.*, 1985). Binding protein distribution *in situ* is similar to IGF immunoreactivity, suggesting that the complexed hormone may interact with target cells (Hill *et al.*, 1989). DNA synthesis was enhanced by complexed IGF1 in cultured fibroblasts (Blum, Jenne, Reppin *et al.*, 1989) but contrary evidence suggesting that free IGF1 is required before receptor binding can take place is also reported (Gopinath, Walton and Etherton, 1989).

Hormonal Control of IGF Secretion

Growth Hormone ~ GH is the dominant endocrine stimulus of plasma IGF1 concentrations and there are numerous examples *in vivo* and *in vitro* of IGF1 dependence on GH status. GH treatment of GH-deficient rats (Robinson, Hodgkinson, Spencer *et*

al., 1990), hypophysectomised rats (Wolf, Ingbar and Moses, 1989) and hypophysectomised sheep (Young *et al.*, 1989) elevates plasma IGF1 concentrations. GH also has the same effect on plasma levels of IGF1 in normal sheep (Bass *et al.*, 1991a), cattle (Davis *et al.*, 1987) and pigs (Owens, Johnson, Campbell *et al.*, 1990). Studies *in vitro* show that GH regulates IGF1 synthesis and secretion from the perfused liver (Schwander *et al.*, 1983) and hepatocytes in culture (Binoux, Lassarre and Hardouin, 1982) with the prior GH status of an animal determining basal levels of IGF1 secretion from cultured hepatocytes (Scott, Martin and Baxter, 1985). GH regulation of immunoreactive IGF1 (Davoren and Hsueh, 1986; Glimm, Baracos and Kennelly, 1988) and IGF1 mRNA has also been reported for several tissues (Hynes, Van Wyk, Brooks *et al.*, 1987; Isgaard *et al.*, 1989).

Little is known about the regulation of IGF2 concentrations in plasma. IGF2 is noted as being less dependent on GH than IGF1, and reduced concentrations following hypophysectomy are suggested to be secondary to reduced secretion of the GH-dependent IGF binding protein (Sara and Hall, 1990). Expression of hepatic mRNA for IGF2 does not alter with hypophysectomy in rats, while mRNA in the brain is reduced with hypophysectomy but is not restored to normal following GH therapy (Hynes *et al.*, 1987). Treatment of dairy cattle with GH *in vivo* failed to alter plasma concentrations of IGF2 (Davis *et al.*, 1987), while GH treatment of pigs decreased IGF2 levels (Owens *et al.*, 1990). From limited data it appears that IGF2 does not respond to GH in a consistent or similar manner to IGF1.

Insulin ~ Insulin has been postulated to be a stimulant of plasma IGF1. Hepatocytes from diabetic rats secrete lower levels of IGF1 than normal and this can be partially reversed by insulin treatment of the diabetic animal. Insulin treatment *in vitro* will also stimulate IGF1 secretion from normal hepatocytes (Scott and Baxter, 1986) and insulin-dependent expression of IGF1 mRNA in vascular smooth muscle cells has also been reported (Murphy, Ghahary and Chakrabarti, 1990).

IGF Inhibitors ~ Studies, particularly *in vitro*, rarely demonstrate a depressive effect of hormonal stimuli on IGF secretion. Many compounds have no effect, but the observation that addition of serum from hypophysectomised rats to normal serum decreased cartilage growth *in vitro* led to the suggestion of physiological inhibitors of the IGFs (Phillips, Belowsky and Reichard, 1979). The existence of IGF inhibitors has now been confirmed and they have been partially characterised. The inhibitor protein(s) are of variable molecular weight and are sensitive to degradation by trypsin and heat (Phillips *et al.*, 1979; Baxter, 1986). They are secreted from the liver and antagonise IGF action at the target tissue (Goldstein and Phillips, 1989).

IGF Feedback ~ There are little reported data on whether IGFs participate in negative feedback control of their secretion. Inhibitory effects of IGF1 on GH secretion, which effectively operates as an indirect feedback control, have been discussed earlier (p 12). A cultured cell line from mice which secretes biologically active IGF1, and also binds IGF1 normally, has been characterised as being selectively non-responsive to IGF1 stimulation (Cascieri, Hayes, Kelder *et al.*, 1988). The authors suggest either that prolonged exposure to IGF1 has desensitised the cells at a post-receptor site or that secretion of an unidentified inhibitory substance has taken place.

Effect of Nutrition on the IGFs in Ruminants

In sheep (Bass *et al.*, 1984; Hodgkinson *et al.*, 1987) and cattle (Breier, Bass, Butler *et al.*, 1986; Elsasser, Rumsey and Hammond, 1989) restricted nutrition or periods of fasting reduce concentrations of the IGFs in plasma. IGF1 in both species is clearly reduced (Breier *et al.*, 1986; Bass *et al.*, 1984) and the mechanism is likely to be via reduced secretory capacity. The half life of ovine IGF1 in plasma does not alter when animals are fasted (Hodgkinson *et al.*, 1987), but tissue concentrations of IGF1 mRNA are reduced with fasting in rats (Lowe, Adamo, Werner *et al.*, 1989).

A number of studies have investigated the dietary variables which may contribute to changes in plasma IGF1. Energy content alone does not alter IGF1 in adult cattle (McShane *et al.*, 1989), but restricted dietary content of protein and energy reduces IGF1 as does limited intake of food (Elsasser *et al.*, 1989). In humans, the protein and energy content of the diet is also reported to control IGF1 in plasma (Isley, Underwood and Clemmons, 1983). In young calves, IGF1 concentrations are transiently depressed with weaning from milk to a pelleted diet (Breier *et al.*, 1988c). Reduced IGF1 concentrations are also reported in pasture-fed compared with pellet-fed sheep (Bass *et al.*, 1987). Bass *et al.* (1984) reported a reduction in plasma IGF2 concentrations with fasting in sheep, but a difference was not apparent between pasture-fed and pellet-fed animals (Bass *et al.*, 1987). In rats, nutritional restriction does not reduce plasma IGF2 concentrations or hepatic expression of IGF2 mRNA (Davenport, D'Ercole and Underwood, 1990). Fasting in pigs reduces IGF2 in plasma (Buonomo, Grohs, Baile *et al.*, 1988), so species-specificity may be an important factor in the nutritional dependence of IGF2 in plasma.

BIOLOGICAL ACTION

Until recently, consideration of the biological role of IGFs has primarily consisted of extrapolation of *in vitro* data and correlative studies of IGF concentrations and physiological status (Copeland, Colletti, Devlin *et al.*, 1990; Siddiqui, Blair, McCutcheon *et al.*, 1990). Research in domestic species has not advanced at the same pace as in rodents due to the lack of large quantities of hormone for administration *in vivo*, coupled with the potential problems of limited bioactivity following complexing of the IGFs to binding proteins in plasma (Steele and Elsasser, 1989). Sara and Hall (1990) propose that the IGFs are basically anabolic hormones with the end result of their action on target cells, mitogenic or metabolic and insulin-like or growth-promoting, being determined by target cell physiology and sensitivity. IGF2 has been postulated to be the foetal growth factor, due to there being relatively greater levels of IGF2 mRNA and hormone in tissues

and plasma during gestational development in many species (Heyner, Smith and Schultz, 1989).

Assigning a more definitive role for IGF1 in growth promotion has been attempted with IGF1 replacement therapy in hypophysectomised rats. Schoenle *et al.* (1982) reported that IGF1 therapy enhanced growth to a similar extent as did GH treatment. They measured weight gain, thymidine incorporation into intercostal cartilage and width of the epiphyseal growth plate. Later, recombinant human N-Met IGF1 was found to be relatively ineffective in restoring the growth of hypophysectomised rats (Skottner, Clark, Robinson *et al.*, 1987). The method of IGF1 administration may be important as to whether an insulin-like or growth effect is elicited (Zapf, Schmid and Froesch, 1984). With a bolus injection, the availability of IGF1 to target tissues is increased promptly, promoting hypoglycaemia and reducing amino acids in plasma (Jacob, Barrett, Plewe *et al.*, 1989), but with longer term subcutaneous administration the animal is protected from acute metabolic effects by the IGF1 complexing to plasma binding proteins (Sara and Hall, 1990). Hypoglycaemia was noted by Skottner *et al.* (1987) with bolus administration of N-Met IGF1, but even with subcutaneous infusion the growth effects of this preparation were marginal. IGF1 is also implicated in insulin treatment of diabetic animals which prevents growth retardation (Scheiwiller, Guler, Merryweather *et al.*, 1986), preventing loss of body weight in fasted mice (O'Sullivan, Gluckman, Breier *et al.*, 1989) and reduced protein loss in fasted sheep (Douglas, Breier, Ball *et al.*, 1990).

Enhancement of growth in normal animals with IGF1 treatment has not been demonstrated, but growth retardation by immunoneutralization has been investigated. Immunization of guinea pigs (Kerr, Laarveld and Manns, 1990) and dwarf rats receiving GH treatment (Spencer, Hodgkinson, Bass *et al.*, 1990) with specific antiserum against IGF1 did not alter growth parameters. Compensatory IGF1 production or action in peripheral tissues may have countered the effect of reduced available IGF1 from plasma.

Studies utilising immunoneutralization of IGF2 or IGF2 treatment *in vivo* to alter growth of normal animals have not yet been performed.

MECHANISM OF ACTION

The two main biological actions proposed for IGFs, insulin-like metabolic effects and growth promoting effects, have been widely established with primary cell culture, organ explants and established cell lines (Sara and Hall, 1990). To date, the mechanistic evidence of either action *in vivo* is limited and indirect, therefore the following descriptions are based primarily on selected reports of effects *in vitro*. The phenomenon of receptor cross-binding exhibited by the IGFs and insulin is discussed in Materials and Methods (p 49). In addition to being a useful means of receptor characterisation it may also be influential in determining the nature of IGF effects on target tissues.

Action of the IGFs on Skeletal Muscle

IGF1 enhances DNA synthesis and replication of myoblasts (Beguinet and Smith, 1985), satellite cells (Allen and Boxhorn, 1989) and fibroblasts (Flier, Usher and Moses, 1986). With damage to skeletal muscle, elevated expression of IGF1 mRNA (Edwall, Schalling, Jennische *et al.*, 1989) and IGF1 immunolocalised to satellite cells (Jennische, Skottner and Hansson, 1987) is observed. During repair of the injury the increase in IGF1 is followed by increased DNA synthesis and then the presence of small muscle fibres showing elevated immunoreactivity (Jennische *et al.*, 1987), which suggests that IGF1 *in vivo* is influential in directing the replication of satellite cells. Myoblasts (Ewton and Florini, 1980) and fibroblasts (Adams, Nissley, Kasuga *et al.*, 1983) in culture also undergo stimulated mitosis with IGF2 treatment.

It has become apparent in some systems that IGFs are more potent as differentiating factors than as mitogens (Froesch, Guler, Ernst *et al.*, 1987). There is increased transformation of cultured myoblasts to myotubes with IGF treatment (Ewton and Florini, 1981) and the effect of IGF1 on proliferation of satellite cells is considerably less

than on differentiation (Allen and Boxhorn, 1989). Differentiating effects of IGFs do not arise as a consequence of their mitogenic effects increasing cell density (Florini and Magri, 1989), but possibly a combination of growth factors in the extracellular environment determines whether IGFs will induce cells to replicate, differentiate or remain quiescent (Allen and Boxhorn, 1989).

The anabolic action of IGFs on muscle cells is reflected in the stimulation of glucose uptake (Poggi, Le Marchand-Brustel, Zapf *et al.*, 1979; de Vroede, Romanus, Standaert *et al.*, 1984) albeit at a potency several-fold lower than insulin. Uptake of amino acids or analogues by muscle cells from culture media is also increased in the presence of IGFs (Yu and Czech, 1984; Shimizu, Webster, Morgan *et al.*, 1986; Harper *et al.*, 1987). In contrast, insulin stimulation of net protein gain is achieved only with pharmacological concentrations (Harper *et al.*, 1987; Gulve and Dice, 1989). The anabolic effects of IGFs on muscle proceed both by stimulating protein synthesis and retarding protein degradation (Harper *et al.*, 1987; Gulve and Dice, 1989). Changes in cell sensitivity to IGFs occur with differentiation (Shimizu *et al.*, 1986; Harper *et al.*, 1987) and whether the source of primary culture is foetal or adult tissue (Roe *et al.*, 1989).

Action of the IGFs on Adipose Tissue

The effects of IGFs on adipose tissue are equally as complex as those of GH. DNA synthesis in Swiss 3T3 fibroblasts is stimulated equally by IGF1, IGF2 and insulin in the presence of epidermal growth factor and bombesin (Corps and Brown, 1988). The same cell line also differentiates into adipocytes under insulin or IGF1 control (Smith, Wise, Berkowitz *et al.*, 1988). Together these reports speculatively suggest that the IGFs may have similar mitogenic and differentiating effects on adipose tissue as they have in muscle. More extensively researched are the insulin-like effects of IGFs on adipose tissue. Glucose transport, oxidation and lipogenesis are stimulated by IGFs, again at a lower potency than by insulin (Zapf, Rinderknecht, Humbel *et al.*, 1978). At lower concentrations than those which result in lipogenic activity, the IGFs reportedly are

differentially effective in stimulating lipolysis. IGF2 was found to be a more effective lipolytic factor than IGF1 (Lewis, Molan, Bass *et al.*, 1988), although this has not been substantiated by more recent work (Robinson, Payne and Bass, 1990).

Action of the IGFs on Bone

Stimulation of longitudinal bone growth by IGF1, independently of GH, has been demonstrated (Isgaard *et al.*, 1986) with supporting evidence of a mitotic role for IGF1 from other studies *in vitro*. IGF1 increases the number of small colonies of cultured chondrocytes from the epiphyseal growth plate (Lindahl *et al.*, 1987) and, together with other growth factors, both IGFs increase numbers of cultured osteoblasts (Slootweg, Hoogerbrugge, de Poorter *et al.*, 1990). Growth of erythroid colonies from bone marrow is also stimulated by IGF1 in culture (Claustres, Chatelain and Sultan, 1987). IGF1 and IGF2 additionally have an anabolic influence on bone collagen synthesis. They increase synthesis and retard degradation independently of effects on cell replication. In this system IGF1 is a more potent stimulus than IGF2 (McCarthy, Centrella and Canalis, 1989).

RECEPTORS

The receptor has been defined as a molecule which specifically recognises and binds a hormone, and as a consequence of recognition may lead to other changes which result in the biological response (Cuatrecasas, Hollenberg, Chang *et al.*, 1975). Hollenberg (1986) categorises receptors into two types; receptors proper which recognise a hormone with high affinity and selectivity, and acceptors which perform a message-carrying function but do not generate a transmembrane signal. The mobile receptor theory (Cuatrecasas *et al.*, 1975) proposes that cell membranes and constituent molecules are dynamic and fluid in nature. Receptors are hypothesized to be relatively mobile and, when complexed with a hormone, will alter their affinity for other membrane components. Several models of the theory describe sequential events of a hormone-receptor interaction which are more

complicated than a linear relationship between occupancy and the biological response (Jacobs and Cuatrecasas, 1985).

GH RECEPTORS

GH receptors are the most poorly characterised of receptors for classical hormones (Waters and Friesen, 1979) due in part to the difficulty in identifying a definitive membrane response to the binding of GH (Asakawa, Grunberger, McElduff *et al.*, 1985). However GH receptors have now been identified on many cell types. Early reports of specific binding of radiolabelled GH were that it was predominantly to membrane preparations from liver (Herington and Veith, 1977; Moore, Draper and Hung, 1985). An observation of specific binding of GH to tissues not normally considered to be targets for GH *i.e.* the adrenal gland and reproductive tissues, was made by Posner, Kelly, Shiu *et al.* (1974). These reports have since been supported by the characterisation of GH binding in cultured fibroblasts (Murphy, Vrhovsek and Lazarus, 1983) and chondrocytes (Nilsson, Lindahl, Edén *et al.*, 1989).

Biochemistry of GH Receptors

The GH receptor in liver (Waters and Friesen, 1979; Tsushima, Sasaki, Imai *et al.*, 1980) and adipocyte membranes (Carter-Su, Schwartz and Kikuchi, 1984; Gorin and Goodman, 1984) is a glycoprotein. The receptor complex may be dissociated by reducing agents into receptor units of variable size (Waters and Friesen, 1979; Gorin and Goodman, 1984; Leung *et al.*, 1987) but there is now general agreement that the hormone-receptor complex is approximately 120 to 140 kDa in molecular weight (Donner, 1983; Carter-Su *et al.*, 1984; Asakawa *et al.*, 1985; Herington, Stevenson and Ymer, 1988).

The GH receptor in rabbit liver has been sequenced and is a 620 amino acid protein with a centrally located transmembrane region (Leung *et al.*, 1987). In adipocyte and liver membranes (Baxter, 1985; Gorin and Goodman, 1985) the half life of receptors has been

estimated to be approximately 45 minutes and also to be independent of occupancy by GH (Gorin and Goodman, 1985). Continued protein synthesis is required to maintain binding capacity (Baxter, 1985; Gorin and Goodman, 1985) and it has been postulated that hormone-receptor complexes undergo endocytosis for degradation and termination of the biological signal (Hizuka, Gorden, Lesniak *et al.*, 1981; Eshet, Peleg and Laron, 1984).

It has been suggested that receptor populations for GH are heterogeneous and perhaps related to mediating the varied functions of GH (Hughes, 1979; Gorin and Goodman, 1984). Donner (1980) postulated that post-binding changes in affinity might take place, and high and low affinity binding sites in liver membranes have been reported for cattle (Breier, Gluckman, Bass *et al.*, 1988a). Herington (1986) noted that only a proportion of receptors were disrupted by reducing agents, perhaps reflecting two receptor types or two conformational states of a single receptor. At the cellular level, receptors which are distinct in antigenic characteristics have been found to be localised in membranes and cytosol (Barnard and Waters, 1986; Herington *et al.*, 1988). There are also differences in antigenic properties between membrane receptors in adipose tissue and liver (Barnard, Rowlinson, and Waters, 1990), supporting the suggestion that the complex metabolic effects of GH on adipocytes may be mediated by an adipocyte-specific receptor for GH (Gorin and Goodman, 1984).

The advent of monoclonal antibodies against the GH receptor, in addition to assisting characterisation and establishing structural or functional heterogeneity of the receptor (Breier, 1987), has also assisted with the *in situ* detection of receptors. Immunohistochemical studies have localised the GH receptor in chondrocytes (Nilsson *et al.*, 1989; Werther, Haynes, Barnard *et al.*, 1990). Extensive cellular localisation has also been demonstrated in the reproductive (Lobie, Breipohl, García Aragón *et al.*, 1990a) and gastrointestinal tracts of the rat (Lobie *et al.*, 1990b), and in the skin of the rat, rabbit and human (Lobie, Breipohl, Lincoln *et al.*, 1990c).

The expression of GH receptors alters with ontogenesis. Gluckman, Butler and Elliot (1983) reported the presence of GH receptors in older lambs and adults, but not foetal or neonate lambs. Similar ontogenetic development has been reported in calves (Breier *et al.*, 1988c) and rats (Maes, De Hertogh, Watrin-Granger *et al.*, 1983), while increased GH binding with aging in adult rats has been observed (Takahashi and Meites, 1987).

Hormonal Control of GH Receptors

The observation of decreased receptor concentrations following hypophysectomy (Gorin and Goodman, 1984) and some restoration of receptors with GH therapy (Posner, Patel, Vezinhet *et al.*, 1980) led to the suggestion that GH regulates its own receptor (Baxter, 1985). Increased concentrations of receptors have also been measured in rats with GH-secreting tumours (Baxter, Zaltsman and Turtle, 1982) and in sheep following long term GH treatment (Harding, Coles, Wallis *et al.*, 1987). There are also reports of increased GH receptor concentrations following hypophysectomy which are reduced after GH therapy (Picard and Postel-Vinay, 1984; Messina, Edén and Kostyo, 1985; Maiter, Underwood, Maes *et al.*, 1988a).

As considered by Posner *et al.* (1980), the effect of a hormone on cellular concentrations of its homologous receptor may vary with tissue type and prevailing physiological conditions. Supporting this, Messina *et al.* (1985) suggested that the differences between up-regulating and down-regulating effects of GH on the receptor may be a consequence of species variation, nature of the membrane preparation and quality of the radiolabelled hormone. Certainly, species variation cannot be discounted, but current data are predominantly from the much studied rat model. Both regulatory influences have been reported in rats, but up-regulation is apparent in adipose tissue (Gorin and Goodman, 1984; Gause and Edén, 1986) so a differential sensitivity between and within tissues to the regulatory effects of GH may also exist.

Maiter *et al.* (1988a) proposed that the mode of GH therapy in hypophysectomised rats may be an important factor in whether receptor concentrations in the liver are up-regulated or down-regulated. They observed an immediate down-regulation after a single GH injection. More recently, up-regulation has been demonstrated following an infusion of GH into hypophysectomised rats (Bick, Amit, Barkey *et al.*, 1990). The influence of normal secretory patterns of GH on receptor populations has also been investigated with Bick, Youdim and Hochberg (1989) concluding that an immediate down-regulation of hepatic receptors takes place in association with a GH pulse and that this is followed by up-regulation to restore the cell's complement of receptors before the next GH pulse. It is obvious that the relationship between GH and GH receptors is complex and not fully understood as yet.

Other hormones are also implicated in regulating the expression of GH receptors. Diabetes causes a large reduction in hepatic GH receptors which is reversible with insulin therapy (Baxter, Brown and Turtle, 1980; Baxter, Bryson and Turtle, 1980), but GH receptor mRNA in the liver is not altered by diabetes (Bornfeldt, Arnqvist, Enberg *et al.*, 1989).

Nutritional Regulation of GH Receptors

Nutritional status is an important factor controlling plasma GH, but effects on the expression of GH receptors may operate independently of any receptor interaction with GH. In rats, fasting or a low protein diet reduces GH receptor number (Postel-Vinay, Cohen-Tanugi and Charrier, 1982; Gorin and Goodman, 1984; Maiter, Maes, Underwood *et al.*, 1988b) and expression of GH receptor mRNA in numerous tissues (Bornfeldt *et al.*, 1989; Straus and Takemoto, 1990). A reduction in the number of receptors cannot be prevented by administration of exogenous GH (Bass, Oldham, Hodgkinson *et al.*, 1991b), but may be reversed with feeding (Postel-Vinay *et al.*, 1982; Straus and Takemoto, 1990). It is apparent that the severity of dietary restriction is a determinant of the extent of mRNA and receptor reduction (Bornfeldt *et al.*, 1989).

In cattle, Breier *et al.* (1988a) demonstrated less binding of GH to liver membranes from poorly nourished animals. From considering the binding characteristics of the membrane preparations it was postulated that the difference was due to a high affinity receptor which was present only with good nutrition. A study with sheep (Bass *et al.*, 1991b) has also demonstrated reduced hepatic binding of GH with poor nutrition and shows the partial prevention of binding site loss with GH administration.

IGF RECEPTORS

The IGF receptors have been characterised to a far greater extent than the GH receptor, possibly as a result of applying photo-affinity and affinity cross-linking techniques originally developed for analysing the structure of the insulin receptor (Czech, 1982).

Biochemistry of IGF Receptors

The Type 1 receptor for IGF1 is a disulphide linked glycoprotein with a molecular weight of ~350 kDa. It consists of two extracellular subunits, ~130 kDa in molecular weight, and two transmembrane β -subunits of ~95 kDa (Massagué and Czech, 1982). Molecular weight and physical properties of the receptor are essentially identical to the insulin receptor (Czech, 1982) but there remain distinct differences (Rechler and Nissley, 1985; Sara and Hall, 1990). The insulin and Type 1 receptors arise from polypeptide precursors (Heyner, Smith and Schultz, 1989) and are the products of different genes (Yang-Feng, Francke and Ullrich, 1985). An earlier consideration was that they may have been translated from the same gene and then undergone differential post-translational modification (Czech, 1982) or both be part of the same protein (Kasuga, Sasaki, Kahn *et al.*, 1983) but this has not proven to be the case. Binding to the Type 1 or insulin receptor generates a transmembrane signal in the form of phosphorylation of tyrosine kinase activity located within the β -subunits (Beguinet, Smith, Kahn *et al.*, 1988).

The Type 2 receptor for IGF2 is structurally distinct from the insulin and Type 1 receptors. The receptor is a single chain polypeptide with a molecular weight of ~250 kDa (Czech, Oppenheimer and Massagué, 1983). The majority of the protein is extracellular with a small transmembrane and cytoplasmic region (Sara and Hall, 1990). The mechanism of transmembrane signalling after hormone binding to the Type 2 receptor has not been determined but the molecule does not contain tyrosine kinase activity (Rechler and Nissley, 1985).

Further species of IGF receptors have been postulated (Rechler *et al.*, 1980), although earlier suggestions will have been confounded by the co-existence of both IGF receptors and binding proteins in membrane preparations. Additionally, impure hormone preparations which were used for receptor characterisation may have contained both IGFs (Baxter, 1986; Sara and Hall, 1990). More recently, a receptor which binds IGF1 and IGF2 equipotently has been suggested (Bhaumick and Bala, 1987; Sessions, Emler and Schalch, 1987) and a low affinity Type 1 receptor (Shimizu *et al.*, 1986; Tollefsen, Thompson and Peterson, 1987). Unique Type 1 receptors have been proposed for brain (Shemer, Raizada, Masters *et al.*, 1987) and foetal tissue (Alexandrides and Smith, 1989). Subspecies of the Type 2 receptor have also been suggested (Taylor, Scott and Baxter, 1987).

The IGF and insulin receptors are internalised by cells following hormone binding. This has been demonstrated *in vitro* with a number of binding systems from different tissues (Oka, Rozek and Czech, 1985; Frank, Pardridge, Morris *et al.*, 1986; Sessions *et al.*, 1987). The extent of hormone internalisation, recycling and degradation varies depending on the hormone (Frank *et al.*, 1986). IGF2 appears to be preferentially degraded, although significant quantities are still released intact at the cell surface (Sessions *et al.*, 1987; Hachiya, Carpentier and King, 1986). There is also evidence that the IGF1-receptor complex may be recycled to the cell surface, by-passing lysosomal degradation and without hormone-receptor dissociation (Furlanetto, 1990).

IGF receptors are widely distributed throughout the body, with most tissues containing Type 1, Type 2 and insulin receptors (Sara and Hall, 1990). Fragments of the Type 2 receptor have also been identified in plasma and urine (Causin, Waheed, Braulke *et al.*, 1988). Type 1 and Type 2 receptors have been identified together in membrane preparations from various tissues of which the placenta (Daughaday, Mariz and Trivedi, 1981; van Buuls-Offers, Hoogerbrugge and dePoorter, 1988), neural tissue (Ocrant, Valentino, Eng *et al.*, 1988), embryonic tissue (Bhaumick and Bala, 1987), gastrointestinal epithelium (Laburthe, Rouyer-Fessard and Gammeltoft, 1988), liver (Caro, Poulos, Ittoop *et al.*, 1988; Hartshorn, Scott and Baxter, 1989), kidney (Hammerman and Rogers, 1987) and mammary gland (Disenhaus, Belair and Djiane, 1988) represent a cross section. It is rarely that a tissue will contain only one IGF receptor, as has been established for chicken liver in which only the Type 1 receptor has been identified (Duclos and Goddard, 1990).

The localisation of IGF receptors has also been determined *in situ* using autoradiography with radiolabelled IGFs (Lesniak, Hill, Kiess *et al.*, 1988; Smith, Clemens, Kerchner *et al.*, 1988) and immunohistochemistry using an antibody specific to the receptor (Valentino, Pham, Ocrant *et al.*, 1988). Binding sites for IGF1 and IGF2 have been identified in the brain (Lesniak *et al.*, 1988; Smith *et al.*, 1988) and various peripheral tissues including the reproductive tract, lung and kidney (Valentino *et al.*, 1988; Monget, Monniaux and Durand, 1989). Cultured cell systems are equally as extensive in their expression of Type 1 and Type 2 receptors, these receptors being found in various cell types from vascular tissue (King, Goodman, Buzney *et al.*, 1985), muscle cells (Shimizu *et al.*, 1986), chondrocytes (Trippel, Chernausk, Van Wyk *et al.*, 1988), osteoblasts (Slootweg *et al.*, 1990) and fibroblasts (Adams *et al.*, 1983).

An important approach to assessing the role of IGFs in foetal and neonatal development is the study of the ontogeny of IGF receptors (Baxter, 1986). Numbers of Type 1 receptors in rat muscle are constant during neonatal life before declining to adult levels

(Alexandrides, Moses and Smith, 1989). Type 1 receptors in bovine mammary tissue also alter in number during the peripartum period (Hadsell, Campbell and Baumrucker, 1990), while the Type 1 receptor from foetal brain tissue in humans appears to undergo transition from a low to high affinity state with advancing gestation (Sara, Hall, Misaki *et al.*, 1983). The Type 2 receptor and mRNA in the foetal rat are expressed concurrently with the expression of IGF2 mRNA and show greatest concentrations in the heart and blood vessels (Senior, Byrne, Brammar *et al.*, 1990). In human foetal tissue there is no change in the binding capacity of hepatic Type 2 receptors (Sara *et al.*, 1983) but an increase in affinity and concentrations during latter gestation has been reported for lambs (Owens, Waters, Thorburn *et al.*, 1985). In rats, Type 2 receptor mRNA and receptor numbers in muscle decline rapidly after birth and remain stable through post-natal life (Alexandrides *et al.*, 1989; Senior *et al.*, 1990).

Hormonal Control of IGF Receptors

Using various binding systems *in vitro* it has been observed that the concentration of Type 1 receptors is down-regulated following pre-incubation with IGF1, IGF2 and insulin (Rosenfeld and Dollar, 1982; de Vroede *et al.*, 1984; Corps and Brown, 1988). The reduced binding follows a reduction of receptor number owing to occupancy by the unlabelled hormones (Rosenfeld, Hintz and Dollar, 1982) and is dependent on the length of pre-incubation and concentration of the unlabelled hormone (Rosenfeld and Dollar, 1982). In the rat, binding of IGF1 is reduced in females with GH secreting tumours (Venkatesan and Davidson, 1990) and in hypophysectomised animals (Lin, Blaisdell and Haskell, 1988). Up-regulation of Type 1 receptors in ovarian tissue of hypophysectomised rats is achieved by treatment *in vivo* with follicle stimulating hormone (Adashi, Resnick, Hernandez *et al.*, 1988), human chorionic gonadotrophin and GH (Lin *et al.*, 1988), but in cell culture GH also has a down-regulating effect on receptor number (Watanabe, Rosenfeld, Hintz *et al.*, 1985). Up-regulation of Type 1 receptors by triiodothyronine is associated with changes in receptor affinity as well as number (Pfeifle and Ditschuneit, 1983). Type 1 receptors are not regulated by numerous other growth

factors and mitogens, including epidermal growth factor and transforming growth factor- β (Adashi *et al.*, 1988; Corps and Brown, 1988).

de Vroede *et al.* (1984) reported that Type 2 receptors in cultured muscle were not regulated in number by prior exposure to the IGFs or insulin. This contrasts with reports of Type 2 receptors in hepatocytes and adipocytes being up-regulated by insulin or GH (Czech *et al.*, 1983; Eriksson, Gause-Nilsson, Lönnroth *et al.*, 1990). It may be a consequence of using a specialised cell line for investigations. The mechanism by which up-regulation of Type 2 receptors is achieved has been investigated and it has been postulated that the hormone causes a redistribution of receptors from intracellular sites to the cell surface (Wardzala, Simpson, Rechler *et al.*, 1984; Braulke, Tippmer, Chao *et al.*, 1990). Changes in both the affinity of the receptor (Oppenheimer, Pessin, Massagué *et al.*, 1983) and the rate of internalisation (Oka *et al.*, 1985) have been reported.

Nutritional Regulation of IGF Receptors in Ruminants

Studies on the nutritional regulation of IGF receptors are limited. Recently, Lowe *et al.* (1989) demonstrated an increase in IGF1 binding and Type 1 receptor mRNA with fasting in rats. They considered that this may have resulted from the removal of down-regulating influences of normal plasma IGFs. The positive response of receptor mRNA to fasting reduces the possibility of increased binding being a reflection of reduced receptor occupancy by endogenous hormone. Changes in IGF1 mRNA and Type 1 receptor mRNA with fasting were not coordinated, suggesting distinct controlling mechanisms for each. There was also considerable variability in which tissues responded to fasting and how they responded (Lowe *et al.*, 1989). Additionally, food restriction in rats increases the number of unoccupied Type 1 receptors located in the median eminence of the brain (Bohannon, Corp, Wilcox *et al.*, 1988). The number of Type 1 receptors in cultured fibroblasts fails to show any change with different culture media (Conover, Misra, Hintz *et al.*, 1988). While this does not reflect a nutritional model *in vivo*, it does suggest some insensitivity to the nutritional environment *in vitro*.

AIMS OF THE THESIS

With agricultural species, an understanding of the integrated metabolic and hormonal environment of the growing animal is an important prerequisite for subsequent research to provide a product which is acceptable to consumers. Apart from the observation that GH is an important hormone in the control of post-natal growth in sheep (Young *et al.*, 1989) there is little known about how changes in nutritional status are effected through the GH-IGF axis to alter growth. This is an important issue in a society which is increasingly aware of the health issues associated with consumption of fatty meat or lean meat from animals which have been treated with synthetic growth promotants, including recombinantly produced GH.

Post-natal control of growth through the GH-IGF axis is via a number of hormones and their specific receptors. Plasma concentrations of the hormones are important, but so too are the status of their receptors in target tissues (Spencer, 1985). There is evidence that both GH and IGF receptors are regulated in capacity by their specific hormones (Baxter, 1985; Corps and Brown, 1988) and nutritional status (Maiter *et al.*, 1988b; (Lowe *et al.*, 1989). The experiments in this thesis were designed to investigate the hypothesis that hormonally or nutritionally stimulated changes in GH and IGF receptor capacity play a part in regulating the growth of prepubertal sheep.

CHAPTER TWO:

MATERIALS AND METHODS

INTRODUCTION

The physiological significance of differences in the metabolism of growing animals has led to the realisation that many systems may not be simply hormone driven, but regulated additionally by processes in target tissues (Spencer, 1985; Daughaday, 1989). The development of radioimmunoassay, radioreceptor assay and membrane receptor preparations has allowed the precise measurement of hormone and hormone receptor concentrations. In turn, this has permitted the scientific observation of smaller changes and differences than previously was possible. Biochemical methods of extraction and measurement often do not provide information on the precise location *in situ* of hormones and receptors, which is an important consideration in tissues which are heterogeneous in their cellular composition *e.g.* skeletal muscle and liver. Research in the endocrinology of growth is now at the point where the trigger for stimulation of cell types within a tissue, or even individual cells of a cell type, needs to be investigated. Cultured cell systems *in vitro* provide one model for such investigations, but in many situations the cells become transformed and do not appear to behave as the same cell type may do in an organism *in vivo* (Cascieri *et al.*, 1988).

Histological autoradiography provides information which is not readily available from classical biochemical techniques. The autoradiograph provides a map of 'how much' of the hormone or receptor is present *in situ* and, more importantly, where within the muscle or liver it is located. In conjunction with other physiological data, a profile of metabolic status can be compiled for the tissue under study.

In the animal experiments of this thesis (Chapters 4, 5 and 6) results from autoradiographic measurement of hormone and receptor populations in target tissues, predominantly ovine skeletal muscle, are considered in conjunction with hormone concentrations in plasma and measurements of growth. Plasma concentrations of GH, IGF1, IGF2 and free thyroxine (T4) have been measured by radioimmunoassay. Receptor populations for GH, IGF1 and IGF2 in muscle were measured using microautoradiography and, indirectly, the significance assessed of IGF cross-binding to the heterologous IGF and insulin receptors. The immunological detection of IGF1 in muscle was also achieved using microautoradiography. This chapter describes the protocols used for the laboratory work associated with these experiments.

MATERIALS

For autoradiography, the following hormone preparations were used as both the radiolabelled hormone and unlabelled hormone.

Ovine GH: purified natural ovine GH (preparation oGH13B14) was received as a gift from the National Institute of Diabetes, Digestive and Kidney Diseases and the National Hormone and Pituitary Programme, University of Maryland School of Medicine, Maryland, USA.

Bovine GH: bovine GH, derived from bacteria using recombinant DNA technology, was received as a gift from Genentech Incorporated, South San Francisco, California, USA.

Ovine IGF1: purified natural ovine IGF1 was received as a gift from Dr L. Moore, Wallaceville Agriculture Centre, Ministry of Agriculture and Fisheries, Upper Hutt, New Zealand.

N-Met human IGF1: human IGF1 with an added methionyl residue at the N-terminus, derived from bacteria using recombinant DNA technology, was received as a gift from Dr B. D. Burleigh, International Minerals and Chemical Corporation, Northbrook, Illinois, USA.

Natural sequence human IGF1: human IGF1, derived from yeast using recombinant DNA technology, was received as a gift from Dr A. Skottner, Kabi Vitrum AB, Stockholm, Sweden.

Ovine IGF2: purified natural ovine IGF2 was received as a gift from Dr L. Moore.

Bovine insulin: purified natural bovine insulin (catalogue number I 1882) was purchased from Sigma Chemical Company.

Ovine prolactin: purified natural ovine prolactin (catalogue number L 4876) was purchased from Sigma Chemical Company.

Bovine serum albumin (BSA): purified natural BSA (catalogue number A 7030) was purchased from Sigma Chemical Company.

Bovine GH differs from ovine GH by two or three residues (Wallis, 1973) and human IGF1 differs from ovine IGF1 by only one residue (Francis *et al.*, 1989). It was considered that the residue differences would not affect specific binding of hormone in the autoradiography incubations. The natural ovine IGF preparations are reported to have less than 0.01% contamination of IGF1 with IGF2 and vice versa (Moore, Jones, Lymburn *et al.*, 1989). The N-Met IGF1 preparation is comprised of two conformations of the hormone, only one of which shows significant binding to ovine IGF plasma binding proteins (Hodgkinson *et al.*, 1989a). As a preparative step in the use of N-Met IGF1, the hormone was resolved into the two conformations by reversed phase chromatography and the material with limited binding capacity for ovine IGF plasma binding proteins discarded (Hodgkinson, Napier, Davis *et al.*, 1989b).

The sources of hormones and other materials used in the experimental treatment of animals (Chapters 4, 5 and 6) and the development of autoradiography techniques (Chapter 3) are outlined in the appropriate chapters.

METHODS

IODINATION OF HORMONES AND ANTIBODIES

All radiolabelled tracer used for autoradiography incubations was produced using a method based on that of Salacinski, McLean, Sykes *et al.*, (1981), with Iodogen as the oxidising agent. Iodogen vials were prepared the day before iodination by placing a solution of Iodogen in dichloromethane (80 µg/ml, 100 µl) in them and storing them uncapped in a drawer to allow the dichloromethane to evaporate. While GH has proven to be a robust molecule with iodination procedures having apparently little effect on GH binding activity (Mattera and Dellacha, 1982; Goodman and Levy, 1983), Salacinski *et al.* (1981) claim that iodination of GH and other proteins with Iodogen gives a product with greater ^{125}I incorporation and less iodination damage than iodination using either chloramine-T (Greenwood, Hunter and Glover, 1963) or lactoperoxidase (Marchalonis, 1969). Iodination of IGF1 using Iodogen results in a product which shows greater than 80% binding to ovine IGF plasma binding proteins (Hodgkinson *et al.*, 1989b), indicating that there is also little damage to the conformation of IGF1 using this procedure.

GH and Antibody Iodination

The following reagents were placed in the Iodogen vial; phosphate buffer (0.05 M pH 7.4, 20 µl), purified antibody (80 µg) or GH (12 µg), and Na^{125}I (1 mCi, 10 µl). This was mixed with a pipette tip and allowed to react for ten minutes, with remixing after five minutes. The reaction was stopped by transferring the contents of the Iodogen vial to a second vial containing phosphate buffer (200 µl). Phosphate buffer with 0.25% BSA (PBSA) was added (200 µl) and the mixture applied to a Sephadex G100 column for purification. The column had earlier been equilibrated with PBSA and fractions were eluted with the same buffer. Aliquots of fractions were counted and the three fractions containing monomeric ^{125}I -GH or ^{125}I -antibody were stored at -20°C for use within three weeks. Specific activities of 73 µCi/µg GH (S. Hodgkinson, *personal communication*)

and 11 $\mu\text{Ci}/\mu\text{g}$ antibody as previously reported (Hodgkinson, Landon and Lowry, 1984) were typically achieved using this procedure.

IGF Iodination

To prepare reversed phase Sep Pak columns for purification of iodinated IGF the columns were equilibrated before iodination. A solution of ethanol (80%) in priming buffer (0.01 M HCl, 0.15 M sodium chloride) was washed through the Sep Pak (2 ml) followed by decreasing concentrations of ethanol (60%, 40%, 20%) in buffer (2 ml) and two washes of priming buffer alone (4 ml). The washing procedure was reversed from buffer to ethanol in buffer (80%) and the original sequence of washing was repeated.

For iodination, phosphate buffer (20 μl), IGF1 or IGF2 (0.4 mg/ml, 10 μl) and Na^{125}I (1 mCi, 5 μl) were combined and mixed in an Iodogen vial. The reaction proceeded for ten minutes, with remixing after five minutes. Contents of the Iodogen vial were transferred to a second vial containing phosphate buffer (50 μl) and incubated for five minutes. Priming buffer with polypep (0.1%) was added (250 μl) followed by buffer alone (135 μl). The mixture was loaded on the Sep Pak column and washed with priming buffer (6 x 2 ml) and each of 10%, 20%, 30%, 40%, 50%, 60% and 80% ethanol in priming buffer (2 ml). Each wash was collected and an aliquot (10 μl) counted. Reagents and iodinated IGF were separated by the column and eluted with different ethanolic concentrations. The iodinated IGF was eluted with the 40% and 50% ethanol/buffer fractions and stored at -20°C for use within three weeks. A specific activity of 130 $\mu\text{Ci}/\mu\text{g}$ was typically achieved using this procedure as for iodination with ^{131}I (Hodgkinson *et al.*, 1987).

AUTORADIOGRAPHY

Autoradiography is a technique which is composed of several sequential but separate procedures (Figure 2.1) for which descriptions follow.

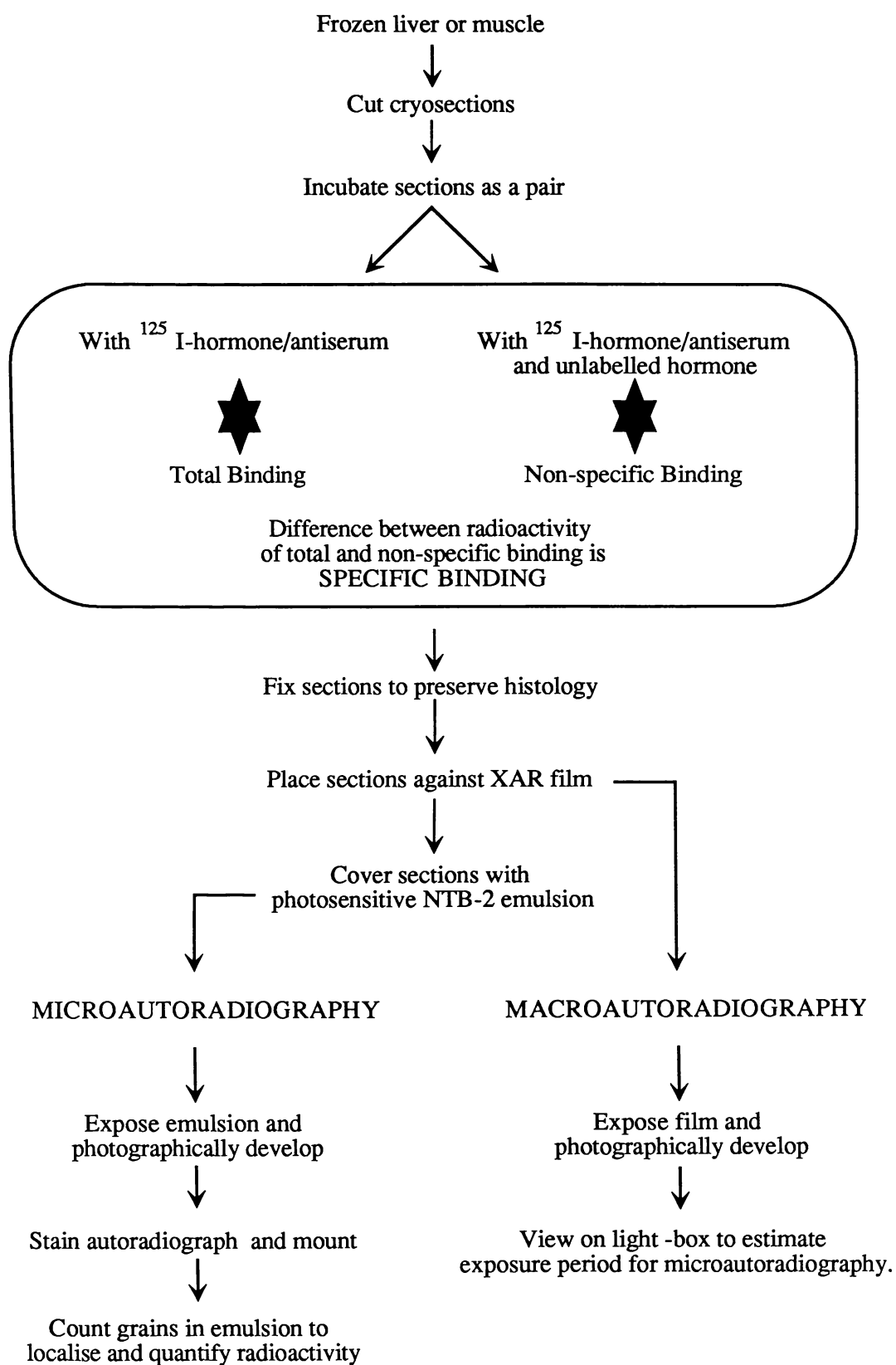


Figure 2.1 Procedure for producing histological autoradiographs following incubation of cryosections *in vitro* with radiolabelled hormone and antibody.

Incubation of Skeletal Muscle Cryosections with Hormone

This protocol is based on that used by Maurer and Reubi (1986) to localise binding of a ^{125}I -SRIF analogue to adrenal glands from rats. Glass slides were cleaned by soaking them in a bath of potassium dichromate (100 g) and sulphuric acid (100 ml) in distilled water (1 l). They were dipped in a subbing solution of gelatine (5 g) and chrome alum (0.5 g) in distilled water (1 l), and dried (Chapman and Hall, 1929; cited in Kodak Limited AR-10 data sheet). Pieces of skeletal muscle were collected at slaughter, frozen in liquid nitrogen and stored at -20°C for periods of up to one year. Cryosections were cut from these samples at -16 to -18°C . The sections were thaw-mounted onto slides and immediately returned to a slide case in the freezing cabinet of the cryostat (Cryo-cut Microtome, American Optical Corporation). They were stored at -20°C until incubated, and used within one week of being cut.

For incubation, sections were thawed and a ring of rubber glue (Ados Chemical Company) placed around them. They were pre-incubated for ten minutes with Tris HCl (0.17 M pH 7.4) and the buffer was aspirated. The incubation buffer was comprised of Tris HCl with BSA (1.0%), magnesium chloride (5 mM) and phenylmethylsulphonyl fluoride (PMSF, 1 mM, Pierce Chemical Company) as the protease inhibitor (Peters and Barrack, 1987). The incubation buffer also contained iodinated hormone (400,000 cpm/ml) or iodinated hormone and unlabelled hormone (range 10 ng/ml to 10 $\mu\text{g/ml}$). The incubation buffer (50 μl) was applied to each section and they were incubated for two hours at room temperature in a closed, humidified chamber. Following incubation the buffer was aspirated and the slides washed in cold Tris HCl buffer with BSA (0.25%) and Triton X-100 (0.1%) (2 x 5 min), fixed in Bouin's fluid (Stefanini, de Martino and Zamboni, 1967) (10 min) and rinsed in distilled water (5 min) before being air-dried.

Incubation of Skeletal Muscle Cryosections with Antibody

Slide preparation, tissue collection and section cutting were the same as for hormone incubations. Sections were thawed and fixed in acetone or ethanol (10 min), then air-dried and surrounded with rubber glue. The slides were pre-incubated for five minutes in Tris buffered saline (TBS) consisting of Tris HCl (0.05 M pH 7.6) and sodium chloride (0.15 M). The buffer was then aspirated and followed by an incubation (20 min) with blocking buffer (normal serum from the same species in which the antibody was raised diluted 1:5 with TBS) to reduce non-specific binding of the antibody. The blocking buffer was aspirated and the sections then incubated for two hours with TBS containing BSA (0.01%) and the antibody alone (400,000 cpm/ml) or the antibody and the antigenic hormone (1 µg/ml) to block the specific binding site of the antibody. Fifty µl was applied to each section and they were incubated in a humidified, closed chamber at room temperature. Following incubation, the buffer was aspirated and the slides washed in a cold buffer of TBS and BSA (0.01%) (2 x 5 min), distilled water (5 min) and then air-dried.

Receptor Cross-binding of the IGFs

As a consequence of the structural homology of the hormones and the receptors, the IGFs and insulin are capable of cross-binding with reduced affinity to heterologous receptors (Van Wyk, Casella, Hynes *et al.*, 1988). Cross-binding is used as a diagnostic tool in biochemical studies of IGFs and insulin tracer binding. The relative potencies of each hormone in competing for binding with the radiolabelled tracer are used to determine to which receptor the tracer is binding. One of the more useful features is that insulin will compete for tracer binding to the insulin and Type 1 receptor, but not to the Type 2 receptor (Nissley and Rechler, 1984). Therefore, the significance of competition for binding by insulin at physiological and pharmacological concentrations can determine whether tracer binding is to Type 1, Type 2 or insulin receptors. This principle has been employed in the work described in Chapters 5 and 6 to determine the type of receptor to which ¹²⁵I-IGF1 or ¹²⁵I-IGF2 is binding.

A basic assumption of binding studies is that the cross-binding of tracer to heterologous receptors is limited by the low concentration of tracer used and that relative affinities of binding sites will dictate tracer binding to the homologous receptor in preference to others. Despite these assumptions, which have proven to be correct in many studies, it is still important to identify receptor type using the competitive potencies of unlabelled hormones. For example, chicken liver membranes do not possess a Type 2 receptor but specifically bind ^{125}I -IGF2. This binding is displaceable by IGF1 and insulin so the binding is concluded to be to the Type 1 receptor (Duclos and Goddard, 1990). Additionally, simultaneous binding of tracer IGF1 to Type 1 and Type 2 receptors has been demonstrated in membrane preparations of foetal skeletal muscle in rats. The much greater concentrations of available Type 2 receptors allowed the binding of ^{125}I -IGF1 to both receptors, despite the ^{125}I -IGF1 having a lower affinity for the Type 2 receptor (Alexandrides *et al.*, 1989).

In several experiments the IGFs have been shown to be several-fold less potent than insulin in competing for ^{125}I -insulin binding (Roth, Maddux, Wong *et al.*, 1983; Alexandrides *et al.*, 1989; Venkatesan and Davidson, 1990). The concentration of 10 ng/ml is within the range of normal physiological concentrations of insulin in ruminant plasma (Blom *et al.*, 1976; Galbraith, 1982) so as an extrapolation of these observations, an autoradiographic incubation of ^{125}I -IGFs (1.4 ng/ml) and 10 ng/ml unlabelled insulin would be likely to demonstrate only specific binding to the insulin receptor.

Similarly, it is reported that pharmacological concentrations of insulin will compete for ^{125}I -IGF binding to the Type 1 receptor, albeit at a potency several-fold lower than that of IGF1 (Roth *et al.*, 1983; Lesniak *et al.*, 1988). A concentration of 10 $\mu\text{g}/\text{ml}$ of unlabelled insulin was selected to compete for binding of the ^{125}I -IGFs to the Type 1 receptor in autoradiography incubations. This concentration was greater than the concentration of homologous IGF (1 $\mu\text{g}/\text{ml}$) used to compete for tracer binding. It was considered that

the demonstration of specific binding with 10 µg/ml unlabelled insulin would reflect ¹²⁵I-IGF binding to the Type 1 receptor. Likewise, failure to show specific binding would suggest that the binding was to a Type 2 receptor. Experiments have shown that insulin in concentrations of up to 1 mg/ml does not inhibit the binding of IGF1 to ovine IGF binding proteins in plasma (Doolin, 1988), so the demonstration of specific binding with unlabelled insulin at 10 µg/ml would not be a reflection of insulin cross-binding to endogenous IGF binding proteins in tissue.

Production of Autoradiographs

Dried slides from antibody and hormone incubations were placed against X-Omat AR5 film (Kodak Limited) in exposure holders for one to five days. Films were processed through an X-ray developing machine (Agfa Gevaert) using Devalex developer and Fixaplast fixer (both May and Baker). Developed films were used to estimate the exposure period for sections under NTB-2 emulsion (Kodak Limited).

Under safelight conditions (Kodak No. 1 filter) in a darkroom, aliquots of NTB-2 emulsion (5 ml) were diluted 1:1 with distilled water and heated to 43°C in a waterbath. The emulsion was applied to sections using the following method. A pasteur pipette was used to deliver a line of emulsion across the width of a slide, between the section and the frosted edge with the label. The tip of the pipette was laid flat on the emulsion and drawn across the section, spreading the emulsion in a layer. The slide was drained of excess emulsion with the labelled end uppermost and dried with dessicant in a light-tight box. The following day, slides were put into plastic slide cases with small trays of dessicant, placed in two black polythene bags and the emulsion exposed to the radioactivity for a suitable period of time at 4°C.

Control sections for latent image fading and chemography were included with each batch of experimental slides (Rogers, 1973). These were sections which were incubated with buffers which did not contain radiolabelled hormone, and then were processed for

autoradiography in the same manner as experimental sections. After emulsion was applied some control sections were exposed to light to monitor latent image fading. This is a phenomenon whereby long periods of emulsion exposure can result in the formation and then loss of a latent image which would normally be developed into a radiographic grain. The remaining control sections were used to monitor the converse problem, that latent image and radiographic grain development might be due to factors other than radioactivity in the section *e.g.* possibly resulting from a chemical interaction between section components and the emulsion. This is termed a chemography control and is also useful to monitor background grain development in the stock emulsion which may increase, depending on age of the product, the temperature of storage, light leaks in storage *etc.*

At the estimated time of optimal exposure, a slide was developed, fixed and stained to assess grain development. For photographic development, slides were equilibrated to room temperature for two hours, developed in D19 developer (Kodak Limited; 3.5 min), rinsed in distilled water (30 s) and fixed in Fixaplust fixer (6 min). The developing and fixing solutions were maintained at 18 to 21°C. Following this, slides were washed in running tap water (20 min) and either dried or stained. When dried, the slides were soaked in distilled water (5 min) before being stained with Ehrlich's haematoxylin and alcoholic eosin. The staining protocol, modified from Doniach and Pelc (1950), is outlined in Table 2.1.

Computer-Assisted Grain Counting

Grain counts were taken from autoradiographs using a computer-assisted system (Figure 2.2). From the microscope (Olympus BH-2) at 1000x magnification under oil immersion, an image was projected through a video camera (Hitachi-kPC), computer-based image processing card (Matrox PIP-512) and on to a monitor screen (Electrohome). Purpose-developed software allowed grains to be counted by manually identifying and marking individual grains using a mouse (Quickmouse) to control cursor

position. Grains were counted within a frame marked on the monitor screen which corresponded to an area of section ($400\text{ }\mu\text{m}^2$). The system could be switched from the live transmission to a fixed image, thus allowing refocussing of the section which was necessary to ensure that all grains within the frame were counted. Visualising of grains directly through the microscope was necessary to distinguish the number of individual grains in clusters, as two or more closely situated grains frequently developed together and the resolution of the monitor was not sufficient to differentiate these.

Selection of the sites for counting was achieved with the use of random numbers and x,y co-ordinates on the microscope stage. Perimeter measurements of the section were made with the co-ordinates, the vertical and horizontal co-ordinates were randomly paired and the microscope set for each pair. For the predominant tissue type in muscle sections (muscle fibre) sites for counting were selected at 1000x magnification. If the counting frame was greater than 85% covered by the tissue of interest, the site was counted. Grains which were on the border of the counting frame were designated as in or out depending on whether the majority of the grain image was within or to the outside of the counting frame.

For tissues which were limited in distribution in muscle sections *i.e.* connective tissue and nerves, a modified system with random numbers was used to increase the chance of site selection for counting. The randomly paired co-ordinates were set and the section viewed at 100x on the monitor screen rather than 1000x. A grid overlay was projected onto the screen (ten vertical and ten horizontal lines, giving 100 crossed points). Points overlying the tissue of interest were counted and the total multiplied by a four decimal random number between zero and one. The answer was rounded to the nearest whole number and determined which point of tissue was moved to the centre of the screen and magnified to 1000x for counting. Using these two systems, six sites for muscle and connective tissue were counted from each section, and up to four sites per section counted for nerves.

Procedure	Time
1. Soak dried, developed slides in distilled water	5 minutes
2. Stain with Ehrlich's haematoxylin (1:3 distilled water)	30 minutes
3. Differentiate in acid / alcohol (0.2% hydrochloric acid : 70% ethanol, 1:4)	1.5 minutes
4. 'Blue' with ammonia in running tap water.	20 minutes
5. Wash in distilled water.	5 minutes
6. Counter stain with alcoholic eosin. (eosin : 95% ethanol, 1:7)	2 minutes
7. Differentiate by passing through 95% and 100% ethanol	
8. Pass through xylene and mount in Depex	

Table 2.1 Method used to stain autoradiographs.
(adapted from Doniach and Pelc, 1950)

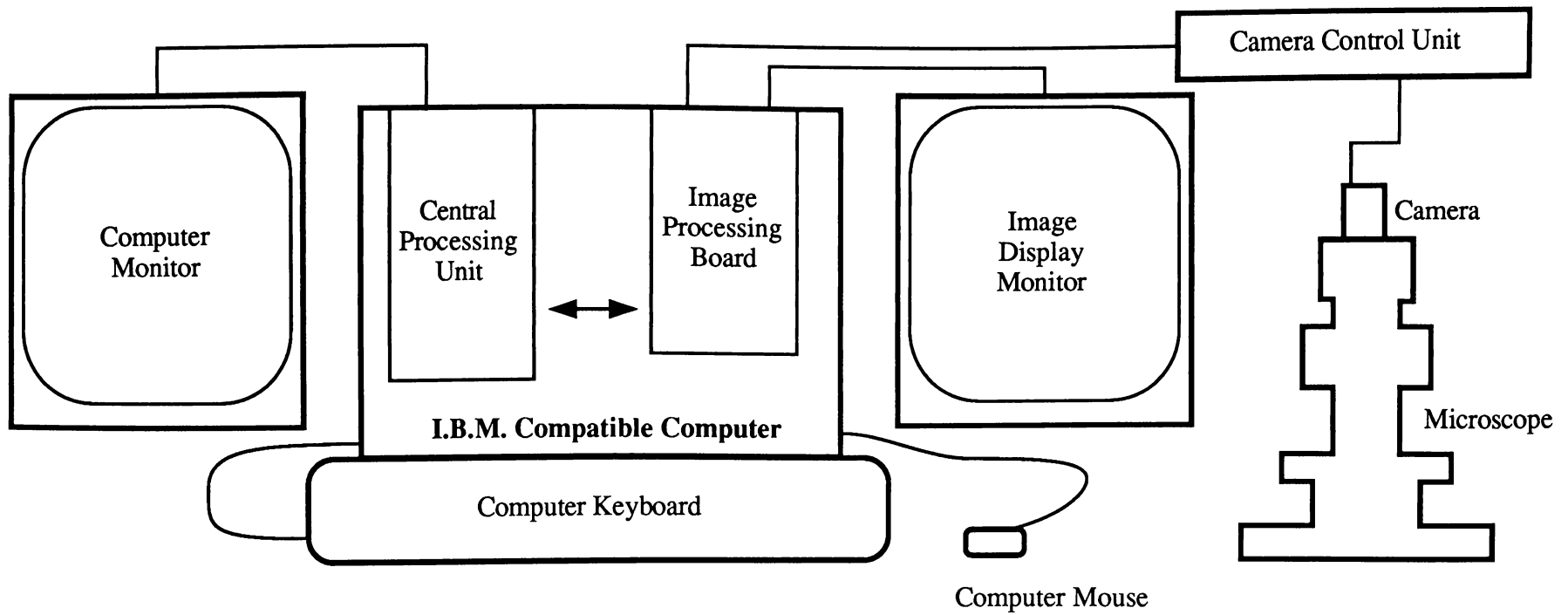


Figure 2.2 The computer system used to count radiographic grains and store data.

PLASMA HORMONE ASSAYS

The radioimmunoassays were performed in two laboratories. For the work described in Chapter 4 the GH and T4 assays were performed by S. Hodgkinson, W. Edgley and G. Lintz of the Growth Physiology and Analytical Biochemistry Groups, Ruakura Agricultural Centre, Hamilton. The assay for IGF1 was provided by Dr. B. Breier and Professor P. Gluckman of Auckland Medical School, Auckland. The GH and IGF1 assays were performed at Auckland Medical School for the work described in Chapter 5, and GH, IGF1 and IGF2 assays provided by the Growth Physiology Group for the work described in Chapter 6.

Radiolabelling of hormones and antibodies in the Ruakura laboratory was performed using the Iodogen method (Salacinski *et al.*, 1981) followed by purification on a gel column. The Auckland laboratory used a lactoperoxidase protocol (Marchalonis, 1969) for iodination of GH and a modified chloramine-T procedure (Greenwood *et al.*, 1963) for IGF1, both followed by purification of the radiolabelled material by gel column chromatography.

GH Assay - Ruakura (Spencer, Bass, Hodgkinson *et al.*, 1991)

Duplicate sample and standard aliquots (100 µl) were incubated with rabbit anti-oGH antiserum (100 µl) and ¹²⁵I-oGH (100 µl, 20,000 cpm) for four days at 4°C. The antiserum was used at a final dilution of 1:13,500 in phosphate buffer with sodium azide (0.1%) and BSA (0.25%). A mixture of goat anti-rabbit antiserum (1:60), normal rabbit serum (1:20) and 4% polyethylene glycol 6000 (PEG) in assay buffer was pre-incubated together for four hours at room temperature and 200 µl added to the tubes which were then incubated for two hours at room temperature. Finally, 500 µl of PEG 6000 (4%) in assay buffer was added, the tubes centrifuged, the supernatant aspirated and the pellets gamma counted. Assay sensitivity is 1 ng/ml with an intra-assay variation of 1.3% and an inter-assay variation of 8.0%.

GH Assay - Auckland (Breier, 1987)

Duplicate aliquots of sample and standard (100 μ l) were incubated with a rabbit anti-oGH antiserum (100 μ l) for one day at 24°C. The antiserum was used at a final concentration of 1:40,000 in an assay buffer of barbitone (0.01 M, pH 8.6) with ethylenediaminetetraacetic acid (0.33%), sodium azide (0.02%) and BSA (0.1%). 125 I-oGH was added (100 μ l, 20,000 cpm) and the incubation continued for a further two days at 4°C. A mixture of sheep anti-rabbit antiserum (0.5%), normal rabbit serum (0.4%) and PEG 6000 (10.0%) in assay buffer was added (1 ml) and the tubes incubated for 15 minutes at room temperature. Finally the tubes were centrifuged, the supernatant aspirated and the pellets gamma counted. This assay has a sensitivity of 0.1 ng/tube with an intra-assay variation of 8.0% and an inter-assay variation of 12.0%.

IGF1 Assay - Ruakura (Hodgkinson *et al.*, 1991)

IGF1 was removed from plasma binding proteins by extracting plasma samples (100 μ l) with 2 ml of a mixture of phosphate buffer (0.016 M pH 7.4, 400 μ l) and cold acid/ethanol (12.5% 1 M HCl and 87.5% ethanol). Tubes were incubated for 30 minutes at 4°C, then centrifuged for 30 minutes at 4°C and the supernatant discarded. Tris base (0.855 M, 1 ml) was added and the tubes were incubated for one hour at -20°C. Finally, the tubes were centrifuged for 30 minutes at -5°C and the supernatant decanted for assay. The supernatant was diluted 1:3 or 1:10 as necessary in an assay buffer of phosphate (0.064 M, pH 7.4), with sodium azide (0.08%) and BSA (0.25%). Duplicate aliquots of extracted plasma or standards (100 μ l), 125 I-IGF1 (200 μ l, 20,000 cpm) and rabbit anti-oIGF1 antiserum (100 μ l) were combined and incubated for 16 hours at 4°C. The antiserum was used at a final dilution of 1:2000 in assay buffer. A mixture of goat anti-rabbit antiserum (1:60), normal rabbit serum (1:200) and PEG 6000 (4.0%) in assay buffer was added to the tubes (200 μ l) and the incubation continued for one hour at 4°C. PEG 6000 (4%) in assay buffer was added (1 ml) and the tubes were centrifuged for 30 minutes at 4°C. The supernatant was aspirated and the pellets gamma counted.

This assay has a sensitivity of 0.2 ng/ml with an intra-assay variation of 3.7% and inter-assay variation of 11.3%.

IGF1 Assay - Auckland (Breier, 1987)

Plasma samples were extracted to remove binding proteins from IGF1 by diluting plasma (50 µl) in 2 ml of a mixture of phosphate buffered saline (PBS, 0.01 M pH 7.5, 450 µl) and acid/ethanol (12.5% 2 M HCl and 87.5% ethanol). Tubes were incubated for 30 minutes at room temperature and Tris base (0.855 M, 1 ml) added to neutralise the extract. Tubes were centrifuged for 30 minutes at 4°C and the supernatant decanted for assay. Duplicate aliquots of extracted plasma and standards (100 µl), and rabbit anti-hIGF1 antiserum (200 µl) were incubated together for one hour at room temperature. The IGF1 antiserum was used at a final concentration of 1:8000 in PBS with ethylenediaminetetraacetic acid (0.37%), sodium azide (0.02%), BSA (0.1%) and protamine sulphate (0.02%). ¹²⁵I-IGF1 was added (200 µl, 20,000 cpm) and the incubation continued for 18 to 24 hours at 4°C. A mixture of sheep anti-rabbit antiserum (0.5%), normal rabbit serum (0.4%) and PEG 6000 (6.0%) in assay buffer (1 ml) was added to each of the tubes which were incubated for 15 minutes at room temperature. Finally the tubes were centrifuged for 15 minutes at 4°C, the supernatant aspirated and the pellets gamma counted. The sensitivity of this assay is 5 ng/ml with an intra-assay variation of 6.0% and an inter-assay variation of 9.0%.

IGF2 Assay - Ruakura (Hodgkinson, Bass and Gluckman, unpublished method)

Plasma samples were extracted to dissociate IGF2 from plasma binding proteins as for the IGF1 assay. A Sep Pak column was equilibrated using trifluoroacetic acid (0.1%, 5 ml, TFA) followed by 2 ml each of polypep (0.1%), ethanol (10%), ethanol (80%) and ethanol (10%) each in TFA, then TFA alone (5 ml). Plasma (100 µl) was diluted with saline (0.9% sodium chloride, 400 µl) and acidified with formic acid (50 µl). The prepared sample was applied to the column and eluted successively with TFA (5 ml), ethanol (10%) in TFA (2 ml) and ethanol (80%) in TFA (2 ml). Dissociated IGF2 was

eluted in the 80% ethanolic solution and then dried under vacuum and reconstituted with ice-cold PBSA (1.5 ml). For assay, duplicate aliquots of the extracted samples or standards (100 μ l) were combined with 125 I-IGF2 (200 μ l, 20,000 cpm) and a monoclonal mouse anti-hIGF2 antiserum (Amano Pharmaceutical Company, 100 μ l). The antiserum was used at a final concentration of 22 ng/ml in PBSA and the tubes incubated at 4°C for two days. A mixture of sheep anti-mouse antiserum (1:34) coupled to mouse IgG with PEG (8%) in PBSA was added (1 ml) and the tubes were centrifuged for 30 minutes at 4°C. The supernatant was aspirated and the pellets gamma counted. This assay has a sensitivity of 30 pg/ml, an intra-assay variation of 5% and an inter-assay variation of 12%.

T4 Assay (Amerlex-M Free T4 Kit, Amersham International).

Duplicate aliquots of plasma and standards (100 μ l) were combined with a 125 I-T4 derivative (500 μ l) and T4 antibody suspension (500 μ l). The T4 derivative was a chemically modified molecule which did not bind to endogenous T4 plasma binding proteins, but was detectable by the specific, high affinity antibody for T4. Tubes were incubated in a waterbath for one hour at 37°C, then attached to a magnetic separation base for 15 minutes. The supernatant was decanted and the tubes inverted to drain before pellet counting. Sensitivity of this assay is 0.1 pM with 3.8% intra-assay variation and an inter-assay variation of 6.9%.

CARCASS COMPOSITION ANALYSIS (Field, Bass, Kirton et al., 1985)

The left sides of carcasses were minced three times through a 4 mm grinder plate. Duplicate minced samples were weighed into extraction thimbles and freeze-dried to determine water content. They were then extracted with petroleum ether to determine fat composition. The dried, extracted samples were placed in crucibles and ashed at 600°C to a constant weight. Finally, protein content was calculated by difference between the measured components - water, fat, ash, and the initial sample weight.

Carcass composition analyses were performed by staff of the Analytical Biochemistry group, Ruakura Agricultural Centre.

STATISTICAL ANALYSES

Data from the grain counting of autoradiographs were analysed by analysis of variance (ANOVA) following the calculation of an average grain count per animal for each tissue type and hormone incubation. To determine the major factors controlling variation of the autoradiography data, estimates of variation with increased sampling were made using data from the GH incubation described in Chapter 5 (p 142). Variance was estimated to reduce by 29% with doubling of the number of experimental animals, 12% with the doubling of sections incubated and 5% with doubling of the number of sites counted. Practical considerations prevented increasing the number of animals or sections for analysis and the limited decrease in variation with more sampling sites on a section did not justify a change in the sampling procedure described above. Autoradiography data are presented in tables as mean counts per unit area of section and in photographic plates. The magnification stated for photomicrographs is the microscope magnification only.

Data from plasma hormone assays were log transformed when necessary and analysed by ANOVA. The ANOVA model assumes that there is normality in the data distribution and that the variance between data points is constant. If the data distribution is skewed or increasing in variance, log transformation can restore the characteristics required for appropriate application of ANOVA. The data presented are back-transformed concentrations (ng/ml) in Figures and Tables. Approximate standard errors of means on the untransformed scale were calculated using the formula $e^m(e^s - e^{-s})/2$ where m is the mean on the log scale and s is its standard error (N.Cox, *personal communication*).

Data from body weight measurements, dissection and carcass composition analyses were analysed by ANOVA without log transformation. These data are presented as weights

(kg) or weight differences (kg and g) from measurement of the same parameters in a pre-treatment group of animals which were slaughtered. When there were differences between body weights of experimental groups at the beginning of treatment, data were adjusted using initial body weight as a covariate. This enabled a clearer demonstration of small treatment effects. Likewise, carcass composition data were adjusted using carcass weight as a covariate when treatment differences in carcass weight were obtained.

The factorial arrangement of treatments used in the studies allows the combination of information on one factor across the levels of the other factor when no interaction is present (Cox, 1958). This increases the precision of the estimates and the means for one factor averaged across levels of the other are called the main effects. All data were tested for main and interaction effects of the experimental treatment of animals, and the Figures and Tables of results include pooled SEMs and statistical significances.

CHAPTER THREE:

DEVELOPMENT OF AUTORADIOGRAPHY TECHNIQUES

INTRODUCTION

The single biggest advantage of using autoradiography and related histochemical techniques is anatomical localisation of compounds *in situ* (Clark and Hall, 1986; Palacios and Dietl, 1987). Measurement of receptors and hormones using autoradiography therefore has considerable potential as a complementary technique for use alongside classical biochemical studies, as membrane preparations do not allow localisation of receptors or hormones to particular cell populations within tissues. The advantages that autoradiography has over other methods of histochemical detection are that it is often technically easier to perform (Ficq and Flament-Durand, 1963) and the data are quantifiable (Budd, 1971).

Depending on the resolution which is required, autoradiographs are produced with film or liquid photographic emulsion. Hahn (1983) used the terminology macro- or microautoradiography to distinguish the two types of autoradiograph. Macroautoradiography uses sheets of high resolution X-ray film for anatomical localisation such as in brain sections (Lesniak *et al.*, 1988) or gland sections (Maurer and Reubi, 1986) and whole body autoradiography (Waddell, Marlowe, Rao *et al.*, 1989). The optical density of the film image, related to radioactivity, may be assessed by eye or with computerised densitometry (Hahn, 1983). The image cast by radioactivity scales, which are standards of known radioisotope content (Clark and Hall, 1986), can also aid the absolute quantitation of macroautoradiographs.

Autoradiographs from histological sections to be viewed using light or electron microscopy are microautoradiographs. They are produced using photo-sensitive emulsion in a liquid form or as a thin layer with gelatin support. The most common quantitative assessment is by using a microscope and counting developed radiographic grains in the emulsion, thus allowing the detection of radioactivity in small areas of section (Clark and Hall, 1986).

Receptor autoradiography *in vitro*, first demonstrated by Young and Kuhar (1979), was notable in that it reduced an earlier dependence on the administration of radioactive material *in vivo*. It also distinguished more clearly between receptor-bound radiolabelled hormone and radiolabelled hormone possibly internalised by cells or physiologically metabolized following administration *in vivo* (Adams, 1980; Clark and Hall, 1986). Successful administration of radiolabelled hormone *in vivo* to measure receptors is limited to compounds or analogues which are metabolically stable (Palacios and Dietl, 1987).

In a comparison of data obtained by autoradiography and radioreceptor assay for prolactin binding, Dubé, Kelly and Pelletier (1980) reported good agreement between relative grain densities in autoradiographs and data from the radioreceptor assay. The sensitivity of assay by autoradiography is at least as good as biochemical assay (Palacios and Dietl, 1987), if not better (Clark and Hall, 1986).

There are no reports to date of histological immunodetection using a radiolabelled antibody against hormones or receptors. More frequently, the antigen is detected by immunoenzymic staining (Dubé *et al.*, 1980; Maddy, Chisholm, Hawkins *et al.*, 1987). While this provides the location of antigen, at very best the quantitation is relative and often based on a system of visual scoring for staining intensity (West, McClellan, Sternfeld *et al.*, 1987). This limits the likely detection of experimental differences to

large changes. Often, immunohistochemistry is performed as a qualitative investigation associated with biochemical assay (Anderson, Ørntoft and Poulsen, 1988).

A recent development in immunodetection has been the use of biotin-streptavidin labelling. Labelling of the primary antibody (direct detection) for immunohistochemistry is a relatively insensitive means of measuring antigenicity. A preferred method is to label the appropriate second species antibody (indirect detection) for incubation following an initial incubation with the antigen-specific primary antibody (Bourne, 1983; Coghill, Swanson Beck, Gibbs *et al.*, 1987). In addition to the increased sensitivity of indirect detection, the highly specific affinity of streptavidin, a bacterial protein, for the small vitamin biotin (Bonnard, Papermaster and Kraehenbuhl, 1984) has been exploited to allow a further intensification of detectable signal. A biotinylated second antibody is used, followed by incubation with labelled streptavidin. This allows significantly greater binding of label to the second antibody without impairing the specific binding capability of the second antibody, and has been used to identify GH receptors on lymphocytes (Bentham, Aldis and Norman, 1990).

Dubé *et al.* (1980) showed a poor comparability of receptor detection by autoradiography and by immunohistochemical staining, but a positive correlation of measurement by biochemical and immunohistochemical assay has been reported for epidermal growth factor receptors (Maddy *et al.*, 1987). Technical considerations for optimal immunodetection often appear to be particular to the antibody in use, so the variation in immunodetection which is reported may be due to these constraints. An advantage of immunodetection over receptor autoradiography using radiolabelled hormone is that detection is often possible with fixed and sometimes paraffin processed tissue. This results in better morphology of sections, potential use of tissues collected for another purpose and a more permanent source of experimental material. The selection of an optimal method of section preparation is a compromise between morphological preservation and *in situ* demonstration of the antigen (Brandtzaeg, 1982).

The aim of the following experiments was to evaluate the suitability of incubation protocols and microautoradiographical techniques for a variety of ovine tissues. The most suitable of these were adapted and modified as necessary for use in conjunction with the animal experiments described in Chapters 4, 5 and 6. Biotin-streptavidin labelling was investigated, as was the use of a protein assay for standardising hormone binding.

METHODS AND RESULTS

PROTOCOL FOR INCUBATION USING IODINATED HORMONE

Section Preparation and Fixation of Ovine Liver

Four methods of tissue preparation, fixation and incubation were assessed for their suitability to use with incubation procedures *in vitro*. Sections of ovine liver were incubated with an oGH-fluorescein isothiocyanate (FITC) conjugate. The choice of a fluorescent label, rather than radioactive tracer, enabled an immediate assessment of binding by fluorescence microscopy.

GH-FITC Conjugation ~ Conjugation of GH to the FITC was performed using a method based on that of Levy and Dawson (1976) for phenylisothiocyanate conjugation to immunoglobulins. Ovine GH (1.4 mg) was dissolved in sodium hydroxide (0.01 M, 500 μ l) and mixed with sodium bicarbonate buffer (0.2 M pH 8.7, 500 μ l). The FITC (2.03 mg, Sigma Chemical Company) was dissolved in sodium hydroxide (0.01 M, 200 μ l) and combined with distilled water (300 μ l). The solubilised FITC was added to the GH in a molar ratio of 5:1 (30 μ l) and the mixture incubated in the dark for two hours at room temperature. β -alanine (10 mM, 100 μ l, BDH Limited) in the bicarbonate buffer was added to quench fluorescence of any dissociated FITC and the incubation continued for 30 minutes. The solution was dialysed overnight in buffer at 4°C and diluted for use. The GH-FITC was diluted in an incubation buffer of BSA (0.25%) in PBS to 10 μ g/ml, wrapped in aluminium foil and stored at 4°C until used.

Section Preparation ~ The liver from one sheep was obtained at slaughter from the Ruakura abattoir. Methods of producing sections were tested which included variables of fixation, the timing of incubation during section preparation and the production of paraffin sections or cryosections. These were categorised into four methods of section treatment as follows:

Liver cryosections; unfixed (control preparation)

- (1) Liver cryosections; incubated and fixed
- (2) Liver piece; fixed, frozen, cryosections cut and incubated
- (3) Liver piece; fixed, paraffin embedded, sections cut and incubated
- (4) Liver piece; unfixed, incubated, fixed, paraffin embedded and sections cut

Tissue for cryosections was frozen in liquid nitrogen and stored at -20°C until sections were cut. Sections were thaw-mounted onto slides, air-dried briefly at room temperature and the drying completed for two hours at 4°C (Nairn, 1969). Sections were then stored at -20°C until incubation. Tissue which was to be paraffin processed was fixed overnight as described below, dehydrated in 100% ethanol (4 x 1 h) and cleared in xylene (4 x 1 h), all at 4°C. The final xylene bath was brought to room temperature and the tissue placed in paraffin baths at 57°C (2 x 1 h) and then embedded in paraffin. The paraffin blocks of tissue were stored at 4°C and sections dried for 30 minutes at 37°C after cutting. For paraffin removal and re-hydration, the sections were passed through xylene (2 x 15 s), 95% ethanol (3 x 15 s) and PBS (3 x 1 min) all at 4°C. This procedure for paraffin processing was developed by Sainte-Marie (1962), particularly for use with immunofluorescence techniques.

If receptor integrity can be preserved throughout paraffin embedding, the advantages include having a more permanent source of experimental material than with frozen tissue. Some protein denaturation occurs during fixation, but localisation of hormone receptors by specific, high affinity antibodies has been successful with paraffin sections (Anderson *et al.*, 1988; Lobie *et al.*, 1990c). Incubation of fixed paraffin sections with hormone, and subsequent localisation with a specific antibody, has also been reported (Petrusz and

Uhlarik, 1973), although others attempting similar localisation have not been successful (Dubé *et al.*, 1980). It was considered that hepatic binding sites for GH might be stable enough to enable detection with labelled hormone after paraffin processing or on fixed cryosections.

Fixation with Bouin's fluid or ethanol was assessed. Salih, Murthy and Friesen (1979) determined that the affinity of prolactin for rat liver receptors was not affected by fixation with a modified Bouin's fluid, while binding capacity was reduced to 65% (Stefanini *et al.*, 1967). Traditionally, cross-linking fixatives such as the aldehydes have been considered unsuitable for retaining the antigenicity of proteins when detection depended on primary structure and conformational features (Brandtzaeg, 1982). Alcohols are extensively used as fixatives for immunocytochemistry, precipitating the protein of interest without affecting immunoreactivity greatly (Grzanna, 1982), so the receptor conformation required for hormone recognition may also be retained. The suitability of ethanol fixation will depend on whether morphology of sections is adequately preserved. Ethanol can cause tissue shrinkage as it fixes by dehydration.

The most suitable time to incubate during section preparation is determined by whether receptor integrity is retained after fixation and/or processing. Fixation before incubation will preserve morphology to a greater extent than fixation after incubation, and paraffin sections show better morphology than cryosections, in which structural damage to the tissue is unavoidably caused by ice crystal formation and thawing (Bancroft, 1984).

Incubation ~ (Nairn, 1969; O. Young, *personal communication*). A drop of the GH-FITC solution (40 µl) was placed over the dry sections, and the slides were incubated for 30 minutes at room temperature in a dark, humidified slide chamber. Excess GH-FITC was removed by rinsing the slides with PBS, then each section was covered with a drop of PBS and agitated on a mechanical shaker for five minutes. The slides were drained, covered with a second drop of PBS and agitated for a further five

minutes after which time they were air-dried to dampness. In accordance with the protocol for paraffin sections (Sainte-Marie, 1962), slides were washed in baths of PBS (4 x 10 min) following incubation. They were then fixed in 95% ethanol for fifteen minutes at 4°C, rinsed in PBS and air-dried to dampness.

Small pieces of liver were incubated for two hours at room temperature in aluminium foil-wrapped vials containing 2 ml of incubation solution. The incubation solution was drained and the tissue bathed in PBS (4 x 10 min) before being placed in fixative for paraffin embedding. All sections were mounted in glycerol buffered to pH 8.0 with carbonate-bicarbonate (0.5 M, pH 9.0) and containing phenylenediamine (1%) to reduce the fading of the fluorescence (Johnson and Nogueira Araujo, 1981; Storz and Jelke, 1984). They were then viewed by epifluorescence microscopy with a Ploemopak (Wild Leitz Limited) mounted on an Orthoplan microscope (Wild Leitz Limited).

Results and Conclusion ~ When viewed by fluorescence microscopy, the control sections showed substantial autofluorescence which made the assessment of the other categories of section preparation more difficult (Figure 3.1). The extent of autofluorescence was such that no greater fluorescent labelling was observed following cryosection incubation and fixation (treatment 1). Sectioning with the cryostat was difficult following fixation and freezing of tissue (treatment 2), and substantially worse following fixation in Bouin's fluid rather than in ethanol. The tissue became brittle and sections shattered as they were cut. With paraffin embedding, there was no evidence of autofluorescence or receptor integrity having been retained throughout processing (treatment 3), the sections showing no fluorescent labelling. Additionally, no fluorescent signal was present on sections from incubated pieces of liver which were subsequently paraffin processed (treatment 4). This may have been a consequence of the time and conditions required for paraffin embedding having led to fading of the FITC label, rather than indicating no binding of the hormone.

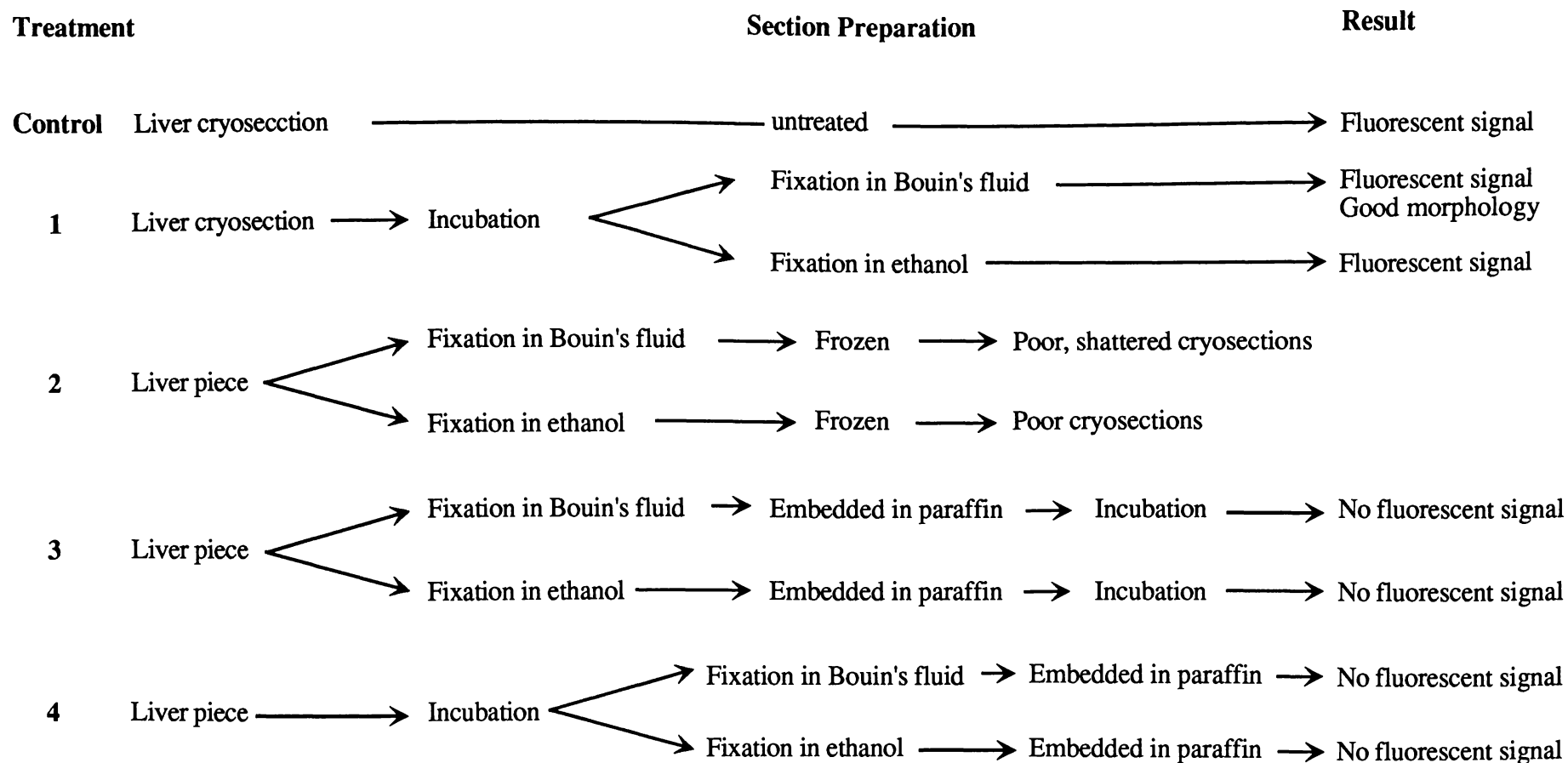


Figure 3.1 Methods of section preparation for incubation *in vitro* of ovine liver with GH-FITC, and results as assessed by fluorescence microscopy.

On the basis of these observations, the procedures which were more suitable (treatments 1 and 4) were selected to re-test using radiolabelled GH. Bouin's fluid was chosen as the fixative as it gave better morphological preservation of tissue and sections after incubation than did ethanol.

Incubation of Ovine Liver and Fat

This trial re-tested treatment 4 (above) and was based on the incubation protocol of Costlow and McGuire (1977) who localised the binding of ^{125}I -prolactin in numerous rat tissues *in vitro*. The aim of the present study was to determine if similar localisation with ^{125}I -bGH was possible in sheep liver and fat, and to determine whether the binding was regulated by prior exposure of the tissue to GH *in vitro*. Regulation of GH binding by GH treatment *in vivo* has been observed in liver (Thissen, Triest, Maes *et al.*, 1990) and fat (Gorin and Goodman, 1984) from rats, and in sheep liver (Bass *et al.*, 1991b).

Materials ~ Alterations were made to the incubation medium used by Costlow and McGuire (1977) to allow the physiological maintenance of ovine liver. The basic medium was comprised of Medium 199, BSA (0.1%), calcium chloride (5 mM) and Hepes buffer (5 mM, pH 7.6). The supplements were sodium propionate as a substrate for gluconeogenesis (10 mM; Elliott, Ash, Pogson *et al.*, 1976), and lactate (10 mM; Clark, Filsell and Jarrett, 1976) and pyruvate (10 mM; Upreti, Riches and Ratcliff, 1991) as intermediates for the tricarboxylic acid cycle. The medium used to incubate ovine fat was Medium 199, sodium acetate (1.1 mM), BSA (1%) and Hepes buffer (25 mM, pH 7.4) as used by Lewis *et al.* (1988). Media were prepared the day before required and kept under O_2/CO_2 (95%/5%) until incubation. Five liver pieces were incubated in vials containing 2 ml of medium, and five pieces of fat were incubated in 4 ml of medium.

Animals and Tissue Preparation ~ Immediately following slaughter, the caudate lobes of liver and samples of subcutaneous back fat were collected from two wether lambs of

one year old. These were placed in their respective media at 39°C and prepared for incubation. Twenty eight pieces of tissue were cut from the fat and liver of each animal. Slices of liver were cut with one border of external connective tissue, and weighed between 30 and 50 mg each. Small pieces of fat were cut which weighed between 15 and 70 mg. Five pieces of tissue were allocated to each of four incubation treatments and the remaining pieces fixed in Bouin's fluid to be embedded in paraffin with the incubated tissue.

Incubation ~ The alternative incubation treatments are outlined below. Each tissue, fat and liver, from each animal was incubated separately at 39°C:

- (1) 3 hour incubation with ^{125}I -bGH (~300,000 cpm/ml, 1.9 ng/ml)
- (2) 3 hour incubation with ^{125}I -bGH plus bGH (1 $\mu\text{g/ml}$)
- (3) 2 hour pre-incubation with bGH (1 $\mu\text{g/ml}$) followed by 3 hour incubation with ^{125}I -bGH
- (4) 2 hour pre-incubation with bGH followed by 3 hour incubation with ^{125}I -bGH plus bGH.

Tissue which was pre-incubated with bGH was drained, blotted and immediately transferred to the appropriate incubation medium. At the conclusion of incubation, the vials were placed on ice, the media were aspirated and tissues rinsed with ice-cold medium (4 x 5 min). Four pieces of tissue from each vial were placed in fixative overnight and processed into paraffin using routine histological procedures. The fifth piece of incubated tissue was blotted, weighed and gamma counted. Aliquots of the solutions used in histological processing were also gamma counted and showed no significant radioactive contamination, indicating the washing of tissues following incubation to be adequate and subsequent handling of sections to be safe. These data also indicated that there was no significant loss of bound ^{125}I -bGH following fixation of the tissue.

Autoradiography ~ Ten sections (8 μm) were cut from each paraffin block and, after de-waxing and re-hydrating, half were stained with haematoxylin and eosin using the protocol outlined in Table 2.1. After differentiating in 100% ethanol, stained sections were placed with unstained sections in distilled water for ten minutes. Staining of sections before applying autoradiography film is postulated to give better histological definition than staining of the autoradiograph (Stein and Yanishevsky, 1979).

Under safelight conditions (Kodak No. 1), pieces of AR-10 autoradiography stripping film (Kodak Limited) were floated on distilled water for a few minutes. A wet slide was placed in the water under a piece of film and lifted out so the film covered the section and draped around the side of the slide. For exposure periods anticipated to be longer than three weeks, the film was floated on a solution of glucose (20 g) and potassium bromide (10 mg) in distilled water (1 l). This was in accordance with Kodak Limited recommendations and research by O'Callaghan, Stevens and Wood (1969) who showed that bromide loss from floating film was significant and affected the subsequent sensitivity of the film. They countered the loss by placing film on a solution of bromide ions and found the film sensitivity also enhanced by the addition of glucose. Such factors are important for sensitivity to low level radiation, which requires a long period of film exposure.

With the film in place, slides were dried overnight in a light-tight box with dessicant, packed into a slide case with fresh dessicant, placed in light-tight bags and stored at 4°C. At intervals of two weeks, a stained and unstained section from each paraffin block was equilibrated to room temperature and photographically developed and fixed. The developer used was Kodak D19 (4 min), followed by a rinse in distilled water (30 s) and fixation in Devalex (May and Baker, 8 min). Solutions were at 18-20°C and the autoradiographs rinsed in running water (20 min) before drying or staining. The unstained autoradiographs were then stained for microscope viewing. All autoradiographs were dehydrated, cleared and mounted in Depex.

Results and Conclusion ~ Data from the incubated tissues which were weighed and gamma counted (Figure 3.2) showed that the incubation protocol demonstrated specific binding of GH by the liver pieces. Competition for the binding sites of radiolabelled hormone with excess unlabelled GH present during the incubation reduced the bound radioactivity by 42%. Pre-incubation of liver with GH did not markedly affect specific binding but counts for total binding were reduced and for non-specific binding were much more variable. This suggested that liver which was pre-incubated had deteriorated to a greater extent than liver which was not pre-incubated.

A further incubation was then performed with liver to test the effectiveness of an anti-proteolytic agent, Trasylol (Sigma Chemical Company), in preventing deterioration of the liver slices. Pieces of liver were taken from three six month old wethers and allocated to each of four incubation treatments, described below:

- (1) 3 hour incubation with ^{125}I -bGH (300,000 cpm/ml)
- (2) 3 hour incubation with ^{125}I -bGH plus bGH (1 $\mu\text{g/ml}$)
- (3) 3 hour incubation with ^{125}I -bGH and Trasylol (500 kIU/ml; Eisentraut, Whissen and Unger, 1968)
- (4) 3 hour incubation with ^{125}I -bGH plus bGH and Trasylol

The tissue was either gamma counted or processed for autoradiography. Data from gamma counting (Figure 3.3) showed total binding of GH only 20% greater than non-specific binding in this incubation and there was no substantial effect of including Trasylol during the incubation. The variability in total binding was less for tissue incubated with Trasylol, but variation in non-specific binding was comparable between the two incubation conditions. Compared with data in Figure 3.2 non-specific binding was substantially greater, suggesting that variability between incubations could be an important factor in incubation experiments.

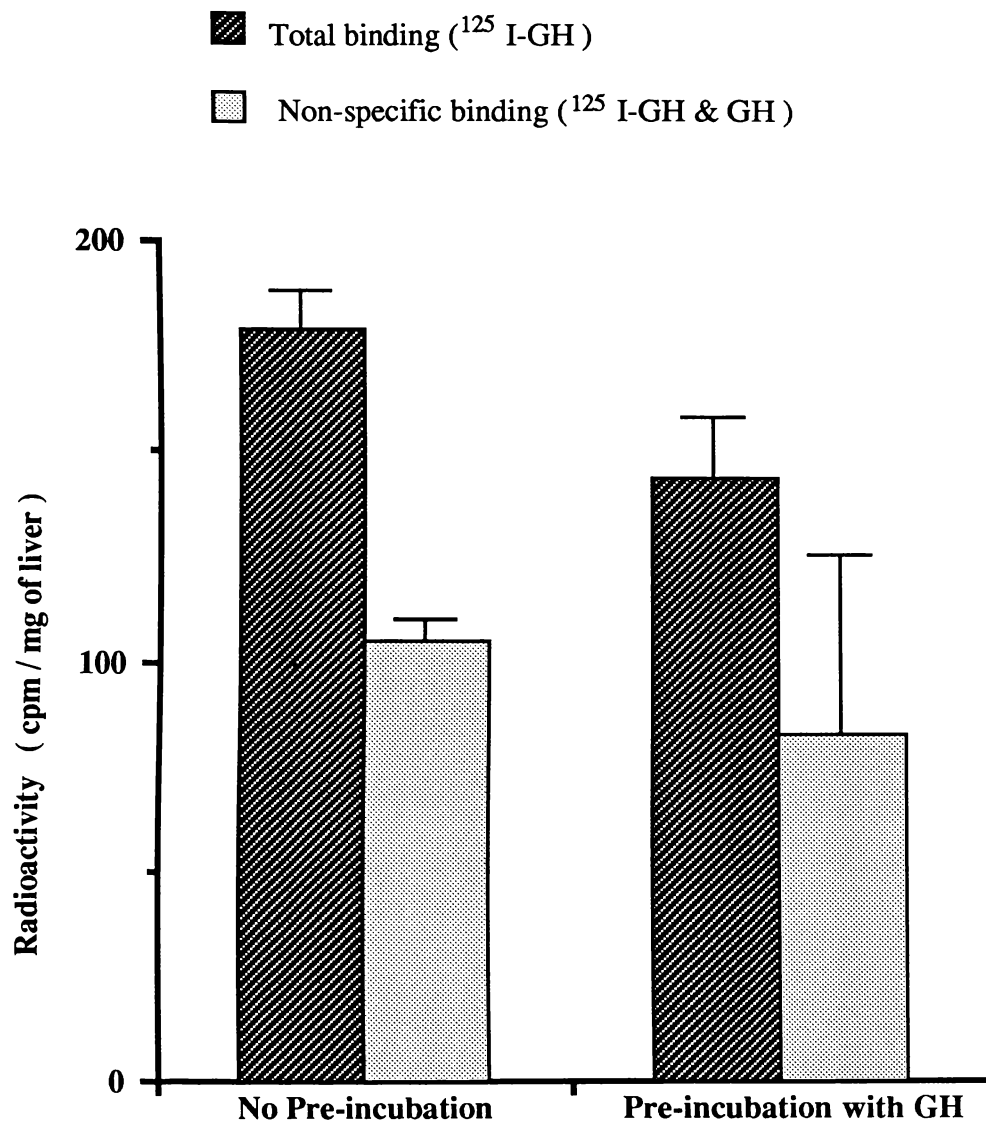


Figure 3.2 Binding *in vitro* of 125 I-GH to pieces of liver, some of which have undergone pre-incubation with GH. ($\bar{X} \pm \text{SEM}$; n=2)

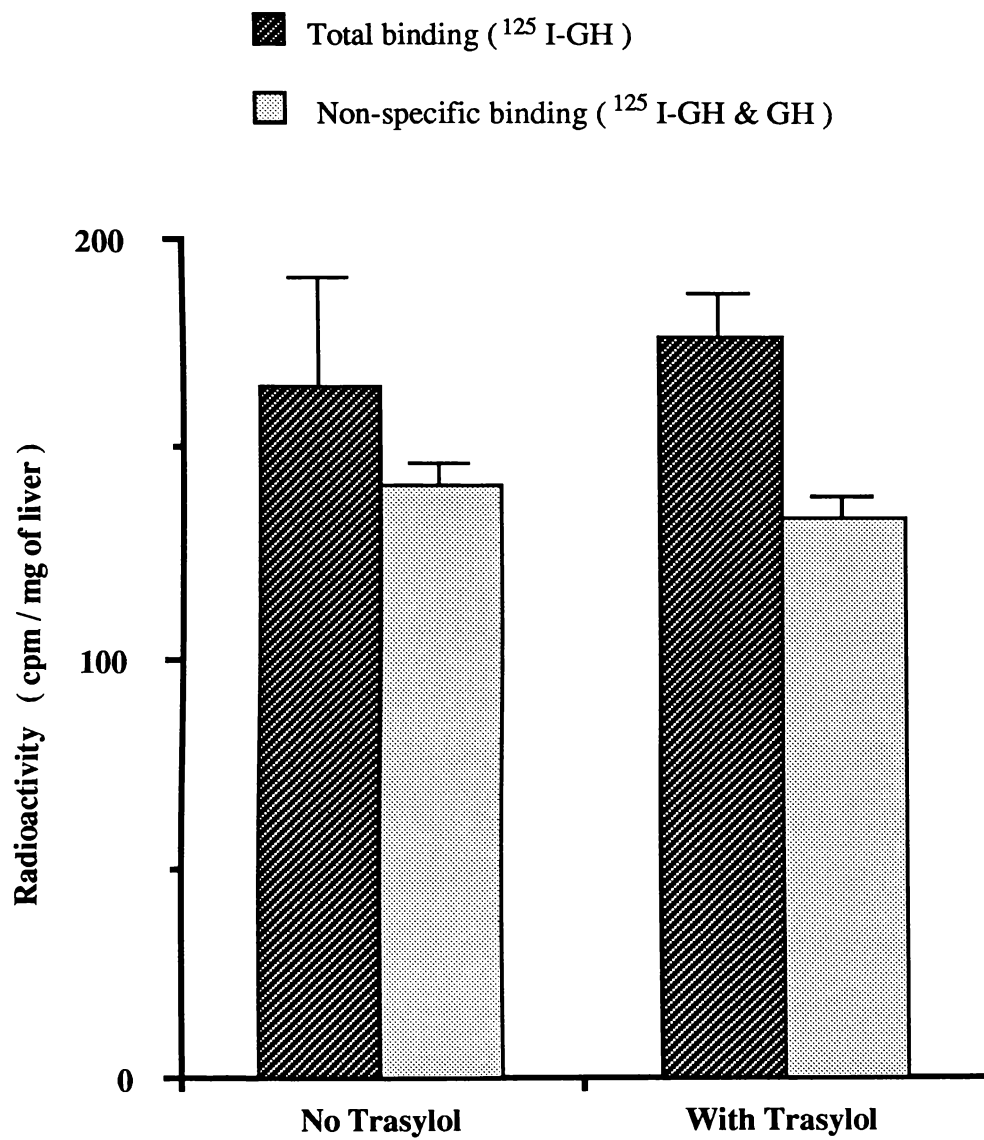
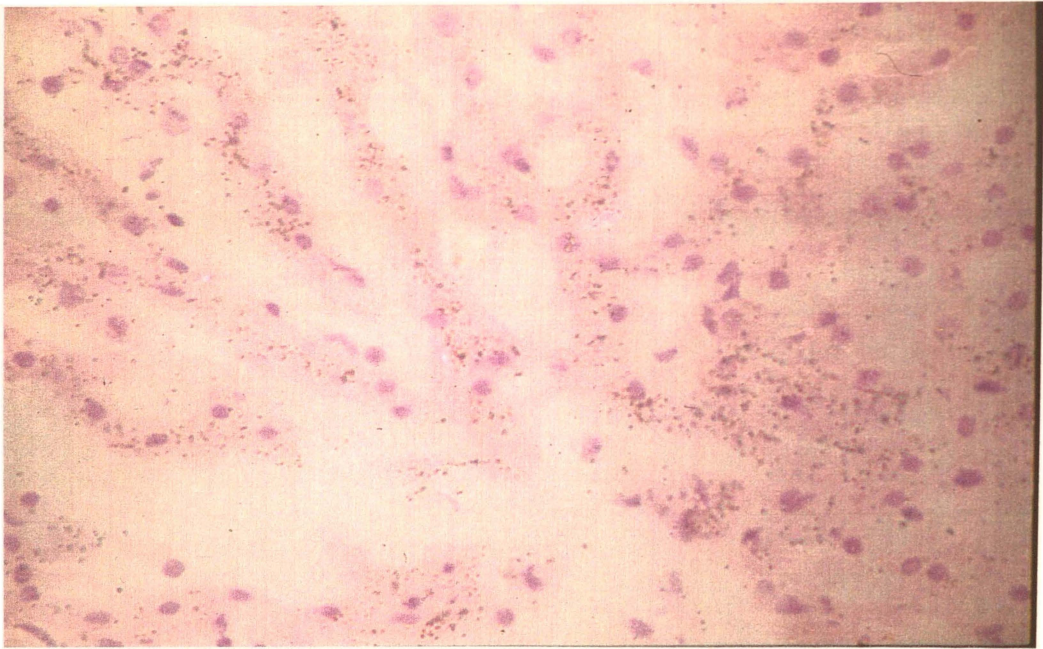


Figure 3.3 Binding *in vitro* of 125 I-GH to pieces of liver, some of which were co-incubated with Trasylol. ($\bar{X} \pm \text{SEM}$; n=2)

A problem was also encountered with analysis of the autoradiographs of liver (Plate 3.1). A pigmented material was present which made the viewing and counting of autoradiographic grains difficult and unreliable. In appearance the pigment was similar to developed grains of the emulsion although slightly larger, often brown in colour and sometimes crystalline in character. It was located in a slightly different focal plane to grains and cells, so was concluded to be at the junction between the section and emulsion. It was not possible to distinguish grains from the pigment reliably enough to pursue grain counting of these autoradiographs. Several treatments, which are detailed in Chapter 4, were subsequently applied to paraffin and cryostat sections of liver in an attempt to remove or prevent the appearance of the pigment in autoradiographs. This trial has indicated that quantitative analysis of liver autoradiographs should be achievable if sections can be treated to ensure the visibility of radiographic grains.

Data from the incubation of fat (Figure 3.4) were inconclusive, with no demonstration of specific binding of GH and no effect of pre-incubation with GH. The morphology of fat cells, where cytoplasmic and nuclear components comprise only a small proportion of the cell body (Lewis, 1987), means that the number of cells per unit weight is variable and dependent on the extent of lipid storage within cells. Quantitation of specific binding by grain counting of microautoradiographs (Plate 3.2) would be inaccurate unless areas with a large component of lipid vacuole were excluded or cross sections of entire cells counted. The anticipated difficulties in accurately measuring the binding of hormones from autoradiographs of fat resulted in quantitative studies not being pursued with this tissue.

A



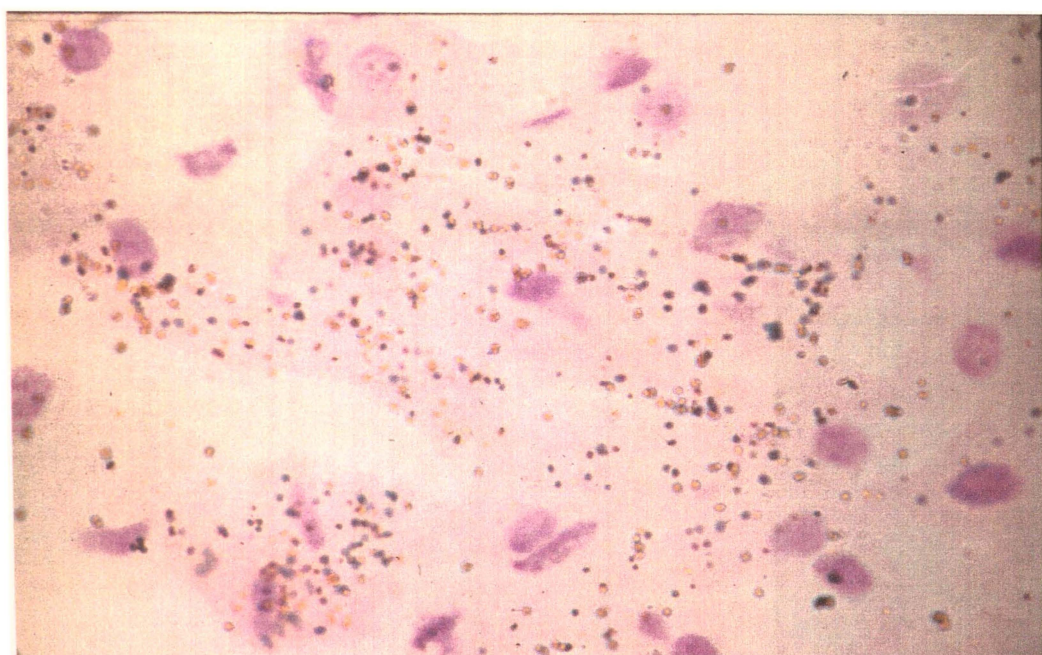
B

Plate 3.1 Microautoradiograph of a paraffin section of liver with AR-10 stripping film following incubation with ^{125}I -GH (A, x400; B, x1000).

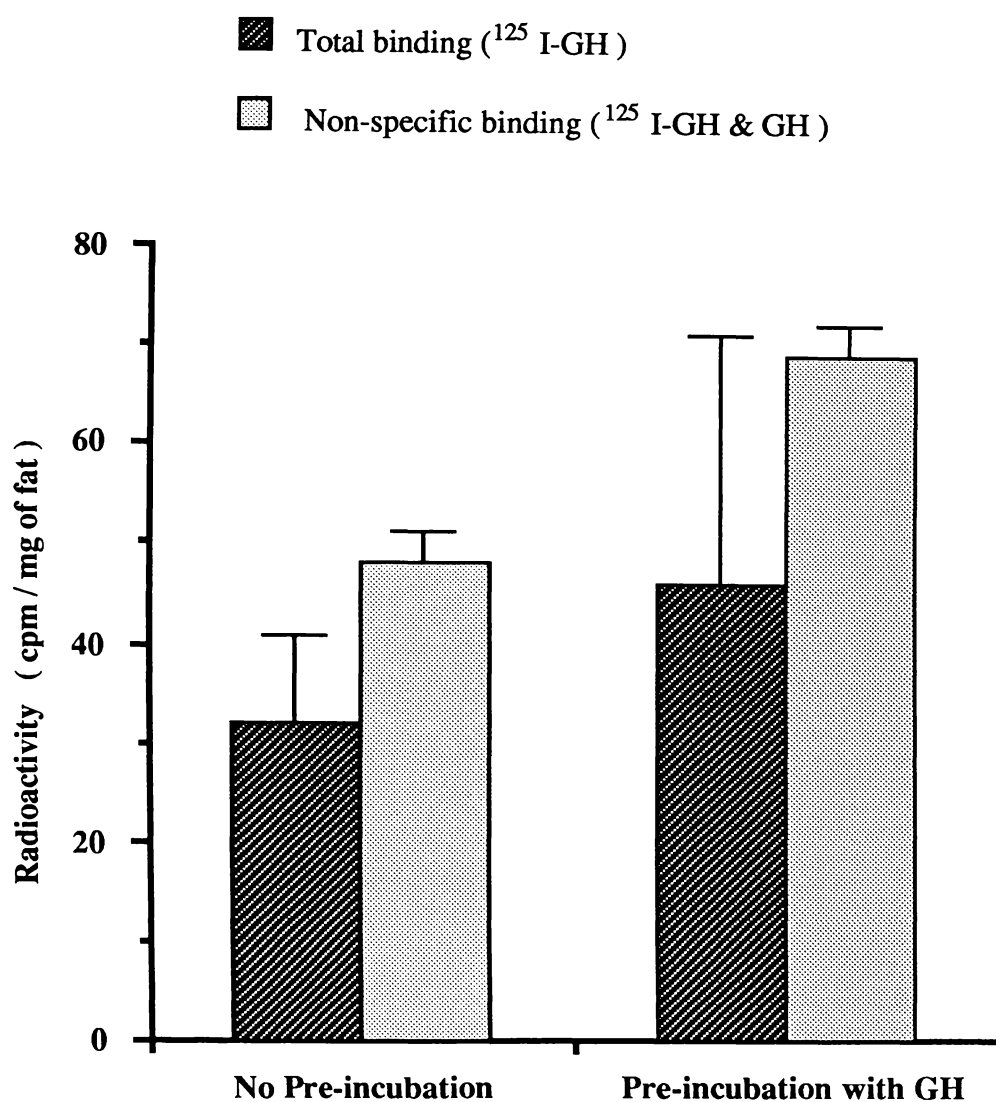


Figure 3.4 Binding *in vitro* of 125 I-GH to pieces of fat, some of which have undergone pre-incubation with GH. ($\bar{X} \pm \text{SEM}$; n=2)

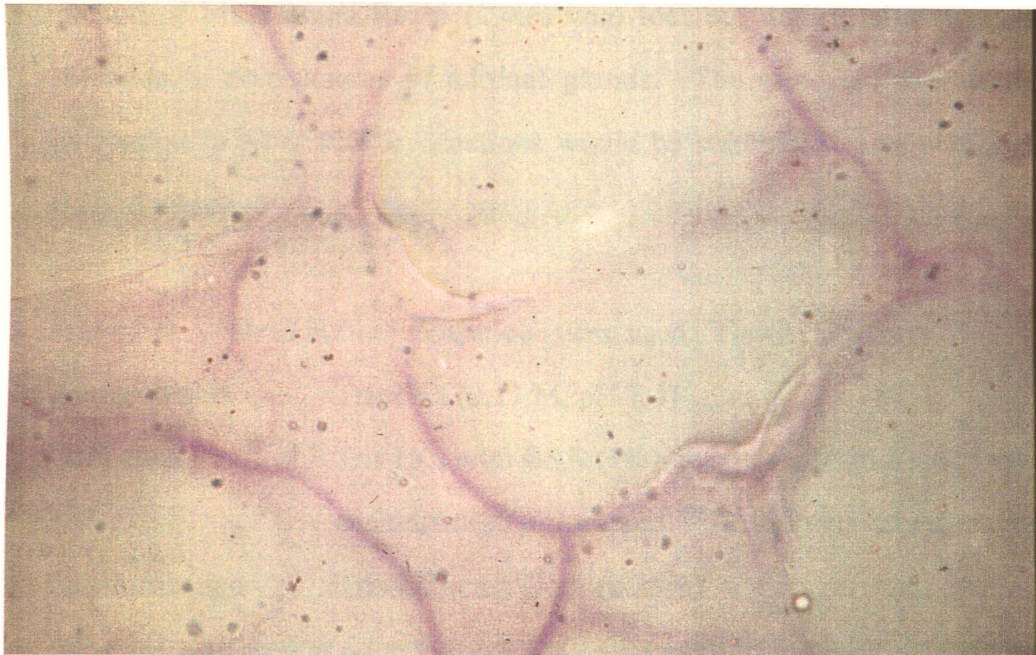


Plate 3.2 Microautoradiograph of a paraffin section of fat with AR-10 stripping film, after incubation with ^{125}I -GH ($\times 1000$).

Staining of slides before processing for autoradiography gave poor morphological definition of cells in the sections. Although slides were overstained as a precaution against stain loss during further processing for autoradiography (Kopriwa and Le Blond, 1962), no haematoxylin was retained in sections by the time they were mounted and viewed. A preference for staining of sections after autoradiography with AR-10 film has been noted by others (Pelc, 1956; Kopriwa and Le Blond, 1962).

Incubation of Ovine Liver and Skeletal Muscle

This trial re-tested treatment 1 of section preparation (earlier) and was based on the method of Maurer and Reubi (1986) who located binding sites for a somatostatin analogue in cryosections of adrenal glands. The aim was to determine whether incubation *in vitro* with cryosections would be suitable for use with ovine liver and skeletal muscle.

Materials ~ Three buffer preparations were used. These were for:

Pre-incubation - Tris HCl (0.17 M, pH 7.4)

Incubation - Tris HCl with BSA (1%), magnesium chloride (5 mM),
bacitracin (20 µg/ml) and ¹²⁵I-oGH with or without oGH

Post-incubation - Tris HCl with BSA (0.25%)

¹²⁵I-oGH was used at an activity of 300,000 cpm/ml with ~500-fold excess unlabelled oGH at 1 µg/ml. This was higher than the radiolabelled hormone concentration used by Maurer and Reubi, but less than used by others incubating iodinated protein hormones with cryosections (Dubé *et al.*, 1980; Chabot, St-Arnaud, Walker *et al.*, 1986). The buffers were prepared the day before use and stored at 4°C.

Animals and Tissue preparation ~ The caudate lobe of liver and samples of the *M. semi-membranosus*, a hind limb muscle, were collected at slaughter from two wether lambs of one year old. The liver was sliced into 5 mm strips and frozen in liquid nitrogen. Small pieces of the muscle were also cut and frozen, and both tissues stored at

-20°C until used. Cryosections (8 µm) were cut at -16 to -18°C and thaw-mounted onto gelatinised slides. They were immediately returned to a slide case in the freezing cabinet of the cryostat and then stored at -20°C until incubated.

Incubation ~ Unlike the method used by Maurer and Reubi, sections were incubated individually (p 48) rather than in jars of incubation buffer. This avoided the use of large volumes of radioactive solutions and the associated problems of possible contamination. Incubating sections individually has also allowed the processing of a large number of sections in a single incubation and has been more conservative in the use of radiolabelled and unlabelled hormone. Sections were thawed and incubated at room temperature. Pre-incubation buffer was placed over them (10 min), then aspirated and followed by incubation buffer with or without the unlabelled GH (50 µl, 2 h). The sections were rinsed in post-incubation buffer and washed in the same buffer (2 x 5 min). To ensure histological preservation throughout photographic exposure and processing for autoradiography, they were fixed in Bouin's fluid (10 min) and washed in distilled water (5 min) before being air-dried.

Autoradiography ~ Two types of autoradiograph were produced from the incubated sections. Initially, the dried sections were apposed to XAR film (p 51) to provide a macroautoradiograph. The film was developed and fixed, and the slides then had NTB-2 emulsion applied (p 51) and were exposed at 4°C for seven weeks. They were developed, fixed, stained and mounted as described in Chapter 2 (p 54). NTB-2 emulsion was used instead of AR-10 film to determine whether the staining of liver autoradiographs might be avoided with this product.

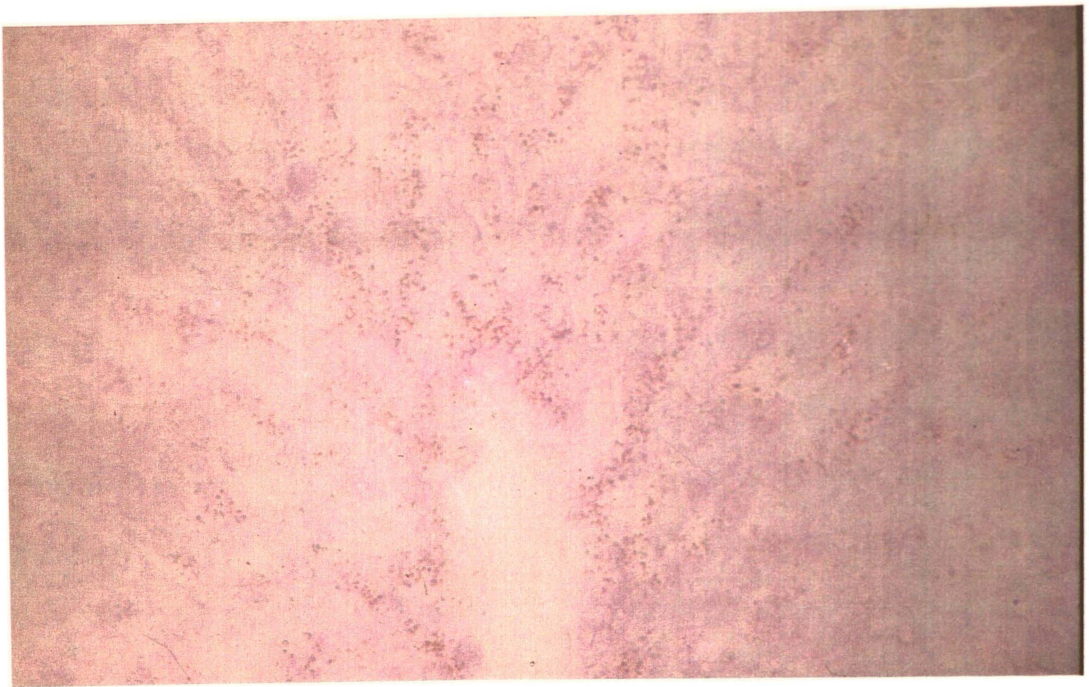
Results and Conclusion ~ This method of incubation for GH with liver sections looked promising after viewing the XAR autoradiographs (not shown). These indicated a qualitative difference in exposure of film between total and non-specific binding treatments of liver sections, and a poor exposure of film for both treatments of muscle

sections. The problem of brown staining encountered earlier with autoradiographs of liver using AR-10 film was not resolved by using a cryostat method of section preparation and autoradiography with NTB-2 emulsion (Plate 3.3). Production of autoradiographs by this method did look suitable for investigating the binding of hormones in skeletal muscle (Plate 3.4). There was reasonable integrity of the sections in the autoradiographs, with staining the various cell populations within skeletal muscle were easily identifiable, and the radiographic grains were clearly seen.

Autoradiography was to be used with tissues from animal trials of a large size ($n = 10-20$), so incubation *in vitro* with cryosections and the production of autoradiographs with NTB-2 emulsion were selected as the routine techniques to be used (Figure 3.5). The techniques were effective at demonstrating hormone binding and the use of cryosections and NTB-2 emulsion was more cost-effective and less labour-intensive than either of their counterparts *in vitro* (incubation of small pieces of tissue and microautoradiography with AR-10 film). This enabled the processing of large numbers of sections in short periods of time which reduces the likelihood of uncontrolled experimental variability in incubation conditions, buffer stocks, radiolabelled hormones, emulsion preparations, *etc.* These findings are in agreement with Kopriwa and Le Blond (1962) who recommended emulsion autoradiography for large volumes of work.

As an added precaution against proteolysis during incubation, PMSF (Peters and Barrack, 1987) was included in the incubation buffer for subsequent work as Trasylol had earlier proven to be ineffective. Also, Triton X-100 was included in the post-incubation buffer to aid the removal of non-specifically bound hormone from sections (Peters and Barrack, 1987). The amount of radioactive hormone used was also increased to 400,000 cpm/ml, a level of activity comparable with other similar studies (Dubé *et al.*, 1980; Chabot *et al.*, 1986).

A



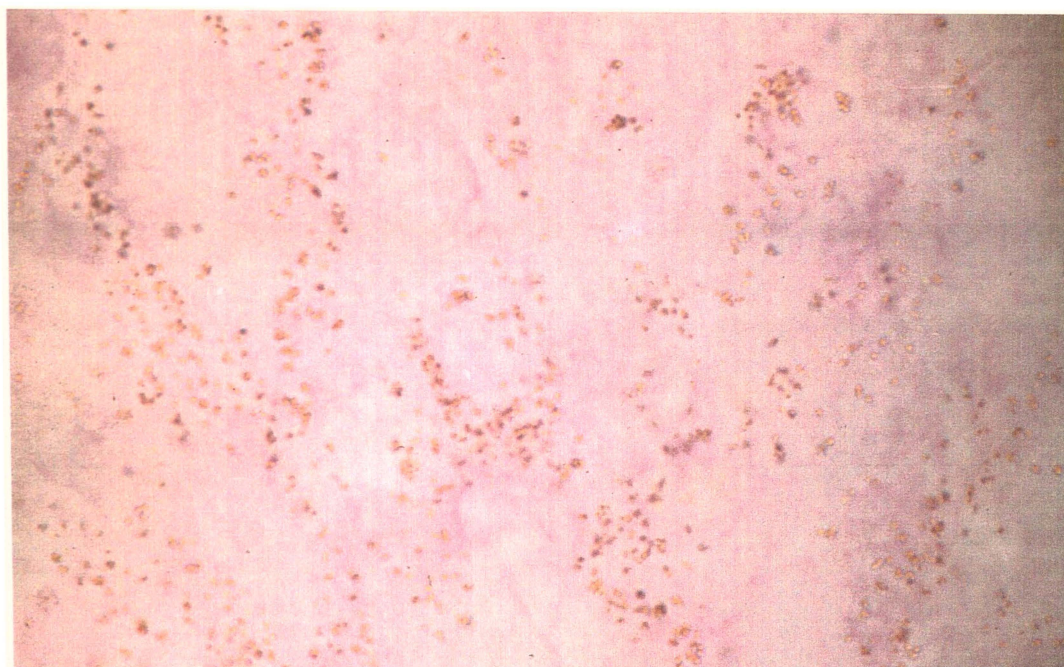
B

Plate 3.3 Microautoradiograph of a cryosection of liver with NTB-2 emulsion following incubation with ^{125}I -GH (A, x400; B, x1000).

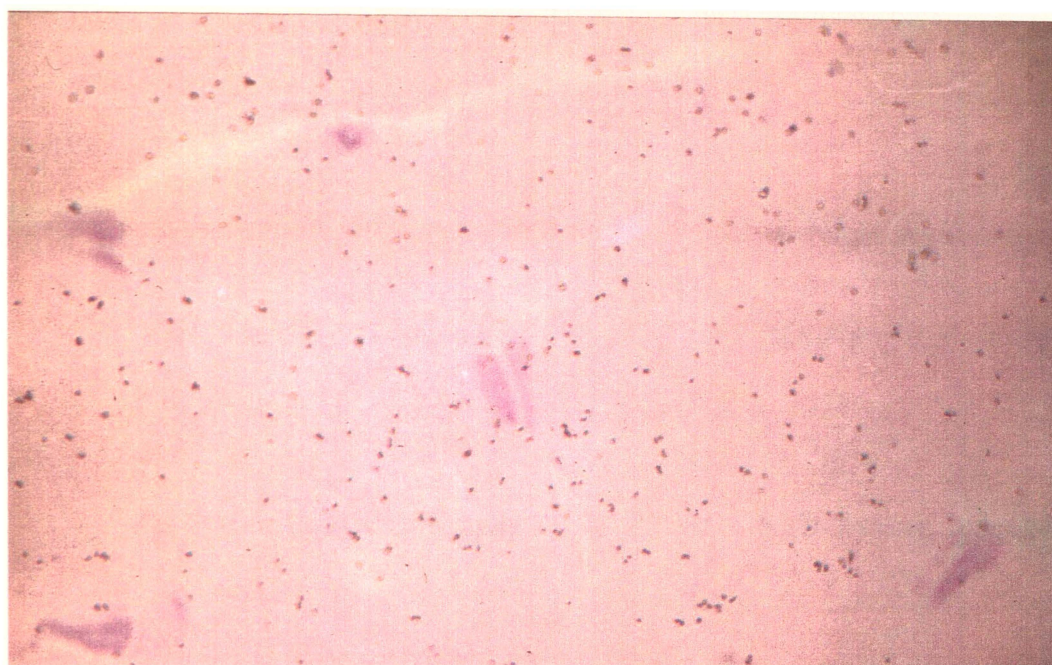


Plate 3.4 Microautoradiograph of a cryosection of muscle with NTB-2 emulsion following incubation with ^{125}I -GH (x1000).

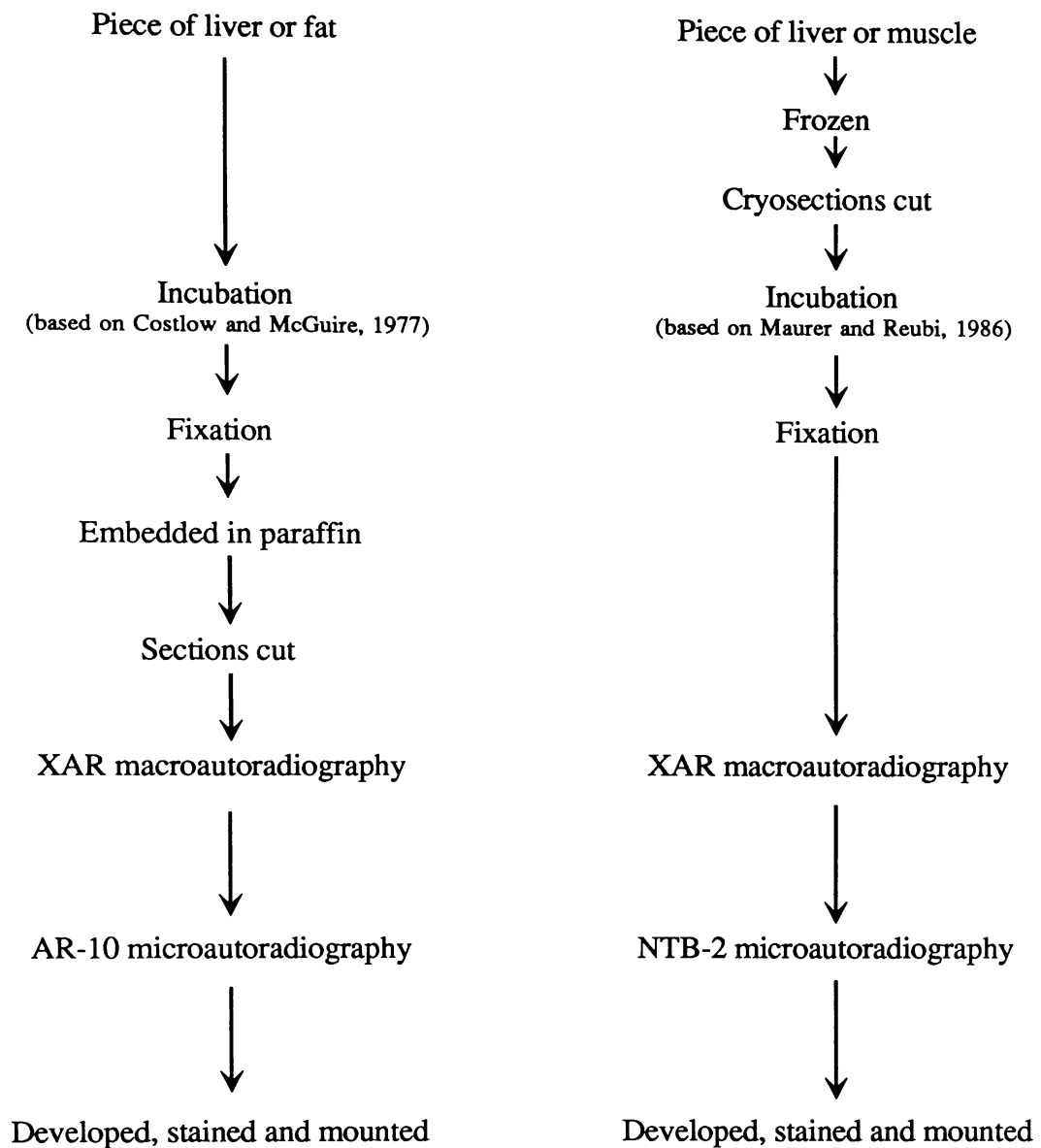


Figure 3.5 Histological autoradiography protocols assessed for incubation *in vitro* of ovine tissues using ^{125}I -GH.

Protein Assay and Gamma Counting as a Predictor of Autoradiography Data

There can be a time lag of months associated with producing and quantitatively analysing autoradiographs before the results of an incubation are known, so an additional incubation protocol for sections was investigated which could provide some immediate information on binding. This would be helpful in assessing the effects of altering incubation conditions or indicating binding differences associated with the experimental treatment of animals. With such low levels of radiation from incubated sections, detection with XAR autoradiographs was not sensitive enough to provide such information. Sections were incubated in tissue culture wells instead of on microscope slides. They were treated as similarly as possible to those used for autoradiography but fixation was omitted and the sections precipitated, solubilised, gamma counted and assayed for protein content.

Materials ~ The buffers used for incubation were the same as those described in Chapter 2 (p 48). Trichloroacetic acid (5%) was used to precipitate sections and a mixture of sodium hydroxide (0.05 M) and Triton X-100 (0.1%) used to solubilise the precipitate (Sainz, Wolff and Dobbie, 1990). ^{125}I -IGF1 was the radiolabelled hormone, used at 400,000 cpm/ml (1.4 ng/ml), and IGF1 (1 $\mu\text{g/ml}$) and insulin (10 ng/ml and 10 $\mu\text{g/ml}$) were used as competing unlabelled hormones.

Animals and Tissue Preparation ~ Extra cryosections of muscle were cut during the preparation for the incubations with ^{125}I -IGF1 which are described in Chapter 5. For each animal, five pairs of sections were cut in total and two pairs randomly allocated to this experiment. They were thaw-mounted to the bottom of tissue culture wells (Multidish, Nunc) which had earlier been gelatinised. Initial investigations showed the necessity of gelatinisation to adhere the section sufficiently throughout incubation and reduce binding of the radiolabelled hormone to the well. Immediately after thaw-mounting, the well plates were returned to the freezing cabinet of the cryostat and then stored at -20°C until incubated.

Incubation ~ Using the same buffers and hormone preparations as for autoradiography the muscle sections were incubated with ^{125}I -IGF1 alone or together with the various unlabelled peptides for 2 hours at room temperature (50 μl). The incubation buffer was aspirated, the sections washed in post-incubation buffer (1 ml, 2 x 5 min) and the buffer aspirated again. Empty gelatinised wells were also incubated to provide data on background levels of protein and radioactivity.

Post-incubation Treatment ~ Following the second post-incubation wash in buffer, the sections were precipitated in trichloroacetic acid (1 ml, 10 min), the supernatant aspirated and the precipitate incubated with sodium hydroxide/Triton X-100 (500 μl , 1 h) at 45°C. The solution was transferred to tubes and aliquots taken for protein assay (2 x 100 μl) and gamma counting (100 μl).

The Biorad protein assay was used to estimate the protein content of each well. BSA standards were prepared in the range of 0-500 $\mu\text{g/ml}$. Duplicate aliquots (100 μl) of standards and samples were pipetted and Biorad reagent (1:4 distilled water) added to each tube (5 ml). The tubes were vortexed and the optical density measured (Gilford, 300 N) during the period from five minutes to one hour following the addition of reagent. Immediately before measurement, individual tubes were briefly vortexed a second time. The protein content of each sample was estimated from the two standard curves using a dual parabolic curve fit to accommodate changes in baseline and sensitivity of the spectrophotometer.

Results and Conclusion ~ The data from gamma counting and protein assay were combined and converted to cpm/ μg of protein and analysed by ANOVA for differences between total binding and non-specific or residual binding. Non-specific binding is the radioactivity which remains following competition for binding by the radiolabelled hormone using excess homologous hormone. Where binding is competed for by a non-homologous hormone, insulin in this experiment, the binding which remains is

termed residual binding. There was a significant difference between total binding and nonspecific or residual binding in all three incubations (Table 3.1, $p < 0.001$), indicating specific binding of ^{125}I -IGF1. These data contrast with the data from autoradiography (Table 5.6) where competition by 10 ng/ml insulin clearly did not result in specific binding.

The accuracy of one or other method of measurement is questionable. In the experiment described in Chapter 5 autoradiography showed ^{125}I -IGF1 binding to be localised to blood vessels and connective tissue in muscle sections. Frequently, these comprised less than 5% of the section area, meaning that most of the section (muscle fibre) was binding ^{125}I -IGF1 non-specifically. The likelihood of detecting such differences between total and non-specific binding treatments with protein assay and gamma counting is limited. Sections in the current experiment contained a maximum of 170 μg protein and bound a maximum of 300 cpm of ^{125}I -IGF1. The small quantities indicated by both measurements necessitates a very sensitive assay to measure specific binding. Protein assay and gamma counting of incubated and solubilised sections is unlikely to provide this when the measurement of interest (specific binding) is calculated by difference. The technique may have applications for assessing the effects of incubation conditions and treatment of sections, and it has subsequently been used to develop an incubation protocol for immunodetection (p 100).

PROTOCOL FOR INCUBATION USING IODINATED ANTIBODY

The demonstration of specific binding with iodinated hormone is a measure of available binding *e.g.* to receptors which are not occupied by endogenous hormone. Detection of endogenous hormones using antibodies is an additional measurement which, in the case of GH will be a direct measurement of endogenously bound hormone in peripheral tissues.

Treatment	Total Binding (cpm / μ g Protein)	Non Specific or Residual Binding (cpm / μ g Protein)	Specific Binding (cpm / μ g Protein)	Pooled S.E.D.	Significance	Residual Degrees of Freedom = 19
¹²⁵ I-IGF1 + IGF1 (1 μ g/ml)	1.4	0.7	0.7	0.11	***	
¹²⁵ I-IGF1 + Insulin (10 μ g/ml)	1.9	1.1	0.8	0.10	***	
¹²⁵ I-IGF1 + Insulin (10ng/ml)	1.6	1.1	0.5	0.07	***	

Table 3.1 Binding *in vitro* of ¹²⁵I-IGF1 to muscle cryosections as determined by protein assay and gamma counting.

($\bar{X} \pm$ SEM ; n=20 animals. *** = $p \leq 0.001$ (ANOVA))

Detection using the Biotin-Streptavidin System

The aim of this experiment was to determine the sensitivity of GH detection using the biotin-streptavidin system in a test tube binding assay. The biotin-streptavidin system of antibody labelling reportedly increases the sensitivity of antigen detection in immunohistochemistry, as discussed earlier (p 64). This experiment tested the conditions required for detection of GH in a gel matrix using a high affinity specific antibody against GH (primary antibody) and a biotinylated second antibody with ^{125}I -streptavidin as the label. The technique, if successful, would be further developed for use with cryosections and autoradiography.

Coupling of bGH to Cyanogen Bromide-Activated Sepharose 4B Gel ~ To provide a homogeneous substrate for antibody binding, bGH was coupled to cyanogen bromide activated gel (Pharmacia), a derivative of Sepharose 4B gel used to immobilise proteins. The gel (2 g) was placed on a sintered glass filter and HCl (0.003 M, 40 ml) added. The suspension was mixed and left for 15 minutes. The HCl was filtered, replaced and mixed, and the washing repeated until 500 ml HCl had been used. Recombinant bGH (20 mg) was added to sodium bicarbonate buffer (0.01 M, pH 8.3, 20 ml) with sodium chloride (0.15 M) and a tracer quantity of ^{125}I -bGH (43,000 cpm, 50 μl). The filtered gel was added to the buffer containing bGH, the suspension mixed gently overnight on a rotating mixer and briefly centrifuged. Aliquots of supernatant (100 μl) were taken for gamma counting and the remainder aspirated. The gel was resuspended in the bicarbonate/salt buffer (5 ml) then centrifuged and the buffer aspirated (3 x 5 ml). The buffer was replaced with bicarbonate/salt buffer containing β -alanine (0.2 M) to block any remaining active groups in the gel. Excess β -alanine was removed using the earlier washing procedure with bicarbonate/salt buffer (5 x 5 ml), then the gel was washed with distilled water (2 x 5 ml) and acetate buffer (0.1 M, pH 4) with sodium chloride (0.5 M) to restabilise it (5 x 5 ml). Finally, the acetate/salt buffer was replaced with phosphate buffer (0.05 M, pH 7.5, 5 ml) containing sodium azide (0.1%) and the gel suspension stored at 4°C until required. The ratio of tracer bGH in the supernatant before and after

bGH immobilisation by the gel indicated 96% incorporation. This gel was termed **bGH solid phase** with 1 μ l approximately equivalent to 1 μ g GH.

Materials ~ The incubation buffer was PBSA. Sephadex G200 (Pharmacia) was used as a gel blank. The primary antibody was raised in rabbits against oGH at Ruakura Agricultural Centre and diluted in PBSA to 1:100, 1:1000, 1:10,000 and 1:100,000. The biotinylated second antibody (Amersham) was raised in goats against rabbit serum and diluted 1:10, 1:100, 1:1000 and 1:10,000 in PBSA containing normal goat serum (1%). 125 I-streptavidin was also purchased from Amersham and used in PBSA at an activity of 200,000 cpm/ml.

Incubation ~ Bovine GH solid phase was pipetted in triplicate into tubes (50 μ l) and G200 gel added (100 μ l). Extra tubes were set up to contain G200 (150 μ l) and 125 I-streptavidin alone (250 μ l). PBSA was added to tubes containing gel and the tubes mixed, centrifuged briefly and the PBSA aspirated (2 x 1 ml). The primary antiserum (200 μ l) at various dilutions was dispensed into tubes and the contents mixed on a rotating mixer (1 h) then centrifuged, the buffer aspirated and the gel resuspended in PBSA (2 x 1 ml). The various dilutions of the second antibody (200 μ l) were dispensed into tubes. The tubes were mixed and washed with PBSA (3 x 1 ml). 125 I-streptavidin (250 μ l) was added to the tubes containing gel and the empty tubes (total counts). Tubes with gel were mixed and washed with PBSA (3 x 1 ml) then centrifuged, the supernatant aspirated and the pellets gamma counted.

Results and Conclusion ~ Maximum binding, 62% of the 125 I-streptavidin added (total counts), was observed with dilutions of 1:100 for the primary antiserum and 1:1000 or 1:10,000 for the second antibody (Figure 3.6). The two concentrations of second antibody show a drop in binding at the same rate with increased dilution of the primary antibody. The non-specific binding of 125 I-streptavidin to G200 gel, 31% of total counts added, was unacceptably high. The profile of the 125 I-streptavidin following separation

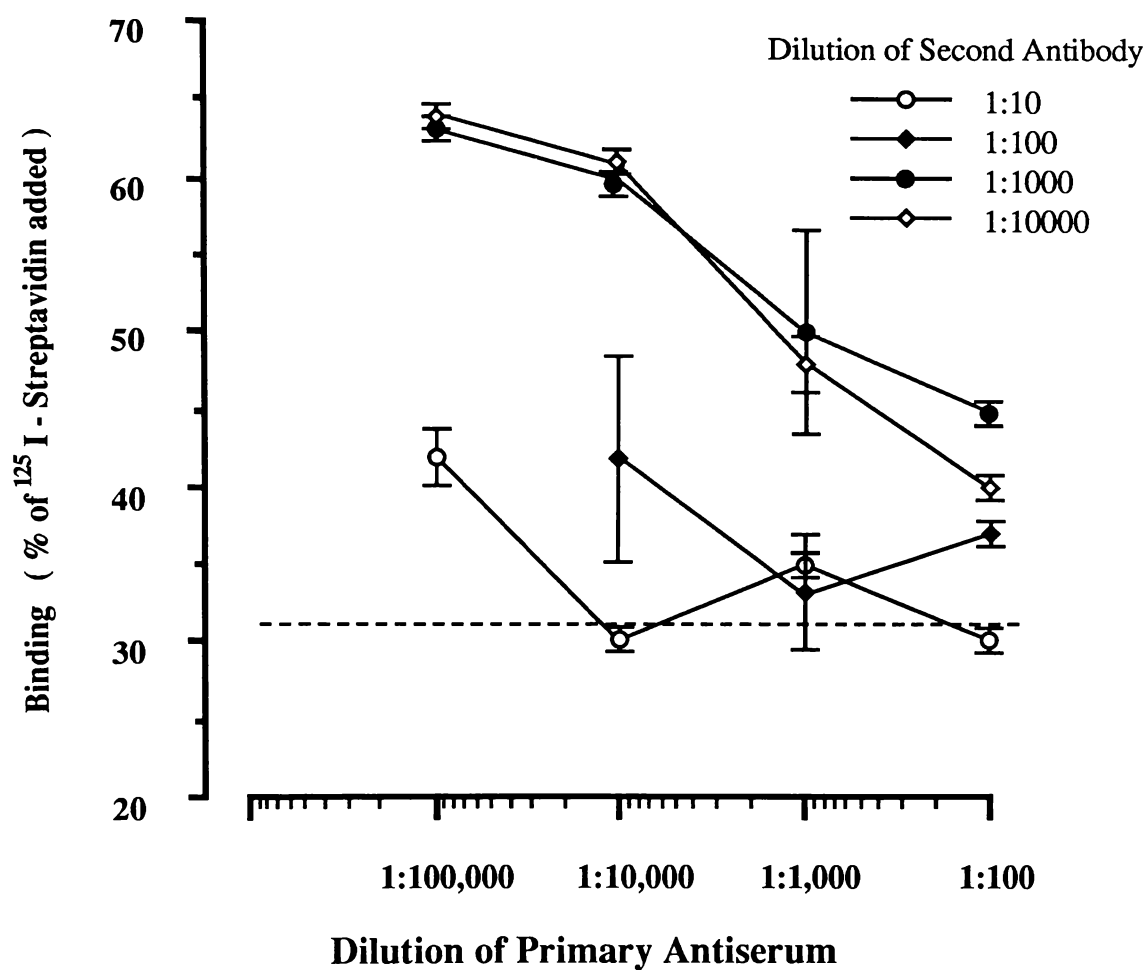


Figure 3.6 Antibody dilution curves used to identify the optimal dilutions of primary antiserum and the biotinylated second antibody for further binding assays. The broken line represents non-specific binding of ^{125}I -Streptavidin to G200 gel. ($\bar{X} \pm \text{SEM}$; n=3 replicates)

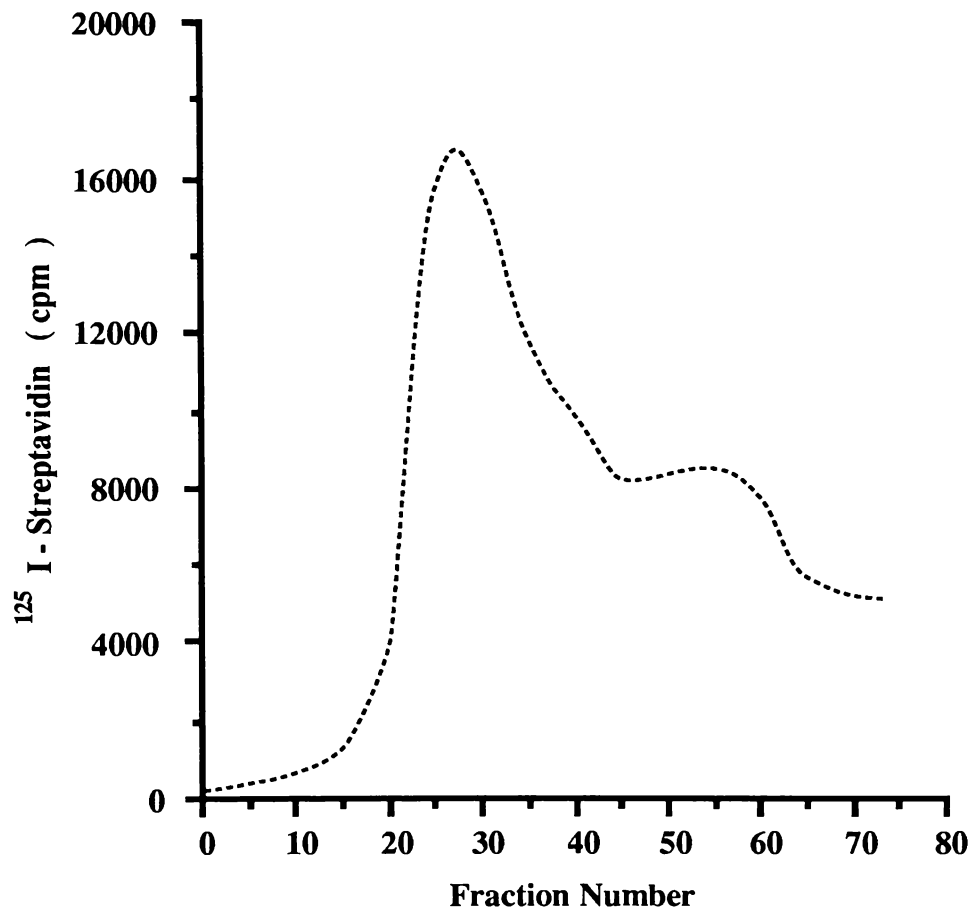


Figure 3.7 Profile of ^{125}I - Streptavidin after separation by fast protein liquid chromatography on a Superose 12 column.

by fast protein liquid chromatography (Pharmacia FPLC System) on a column of Superose 12 (Figure 3.7) indicates the lack of a single major peak of iodinated product, suggesting that degraded products of ^{125}I -streptavidin may have contributed to the high non-specific binding which was observed. Immunohistochemical detection using a biotin-streptavidin system looked possible as a technique if the source of non-specific binding could be identified and reduced. Results have indicated that the ^{125}I -streptavidin may have contributed significantly to non-specific binding.

Investigation of Non-Specific Binding in the Biotin-Streptavidin System

The buffers, the gels, characteristics of bGH, degradation of ^{125}I -streptavidin or a feature of hormone incorporation into the solid phase may all contribute to non-specific binding of ^{125}I -streptavidin. The aim of this experiment was to determine the source of non-specific binding previously observed with use of biotin-streptavidin immunodetection.

Materials ~ Two incubation buffers were used; PBSA as used previously, and sodium bicarbonate buffer (0.01 M, pH 8.3). Additionally, concentrations of sodium chloride were tested with each buffer (0.15 and 0.5 M). The influence of gel type was also assessed; Sephadex G200, Sephacryl S300 and Sepharose 6B (all Pharmacia). ^{125}I -streptavidin was used at an activity of 200,000 cpm/ml in each of the incubation buffers.

Incubation ~ Each of the gels and bGH solid phase was incubated with a buffer/salt solution containing ^{125}I -streptavidin (250 μl). Tubes without gel were also incubated with buffer/salt solution and label, while others contained total counts. Tubes were incubated for an hour and a half with vortexing every 30 minutes. They were briefly centrifuged, the supernatant aspirated and the gel resuspended in buffer/salt (3 x 1 ml), and then centrifuged, the supernatant aspirated and the pellets gamma counted.

Results and Conclusion ~ Results indicated that there were only minor effects of buffer, salt concentration and type of gel on non-specific binding of ^{125}I -streptavidin (Figures 3.8 and 3.9). With PBSA at all salt concentrations, binding to the gels was similar and approximated 3% of total counts. With bicarbonate buffer, there was a trend of increased binding without sodium chloride and with the Sephacryl S300 and Sepharose 6B gels.

In contrast, non-specific binding to the solid phase was high with both buffers and all salt concentrations, ranging between 40% and 54% of total counts. Using the bicarbonate buffer with salt (0.5 M), the incubation was repeated with two other hormones which were coupled to cyanogen bromide activated 4B gel in the same manner as bGH, oGH and recombinant hIGF1. These solid phases showed non-specific ^{125}I -streptavidin binding of 3% and 2% respectively. This indicated that the high non-specific binding of ^{125}I -streptavidin was not due to general properties of buffers and salt concentrations, protein hormones, recombinant products or solid phase preparations. The nonspecific binding was apparently associated with bGH, recombinant bGH or this particular preparation of bGH solid phase.

Detection of oGH using the Biotin-Streptavidin System

The original incubation protocol for biotin-streptavidin detection was retested with oGH solid phase using the primary and second antibody dilutions which gave greatest GH detection previously (Figure 3.6). Additionally, the extent of non-specific binding was assessed at each step of the binding assay.

Materials ~ Primary antiserum was used at 1:100 and second antibody at 1:10,000 with normal goat serum (1%) in PBSA. ^{125}I -streptavidin was used at an activity of 200,000 cpm/ml in PBSA. The solid phase was oGH with Sepharose 6B gel used as a control.

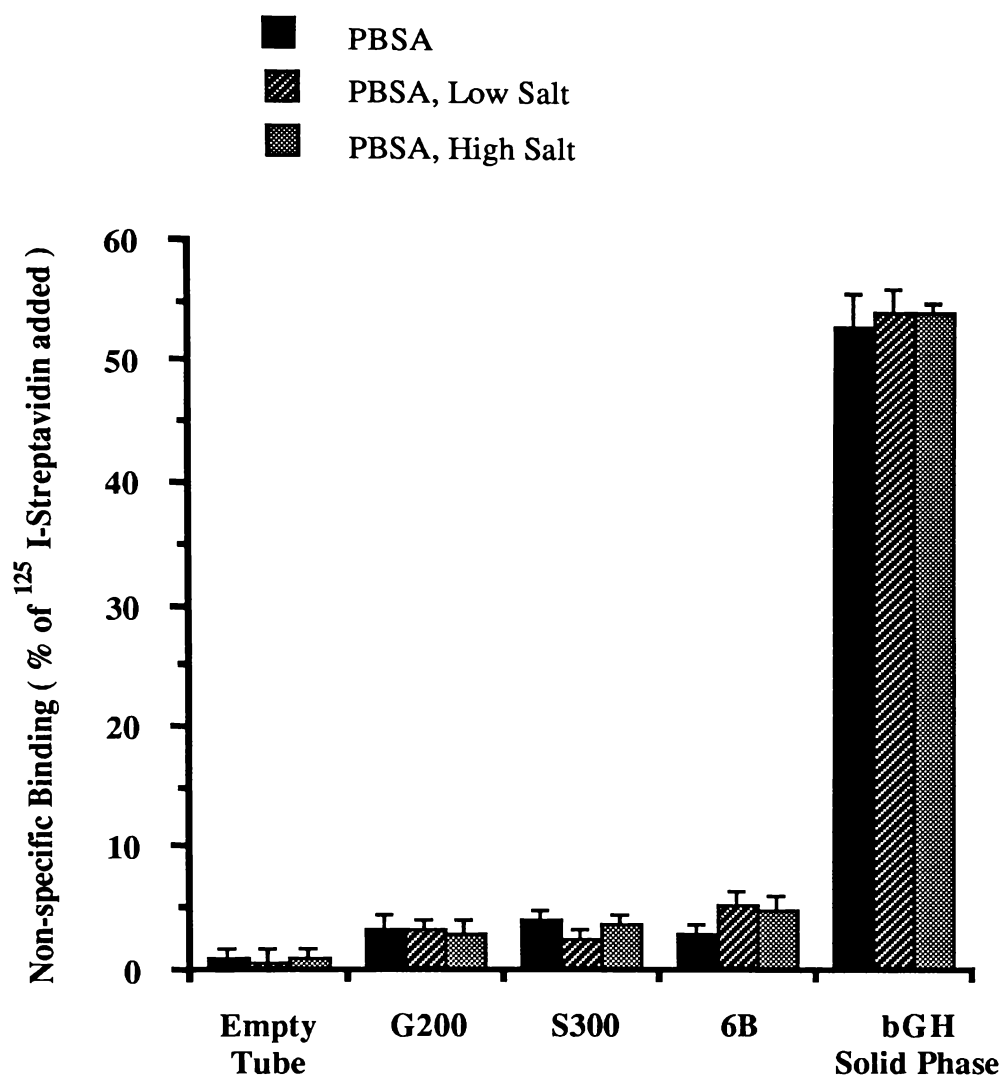


Figure 3.8 The effect of gel and salt concentrations in PBSA on non-specific binding of ^{125}I -Streptavidin. ($\bar{X} \pm \text{SEM}$; n=3 replicates)

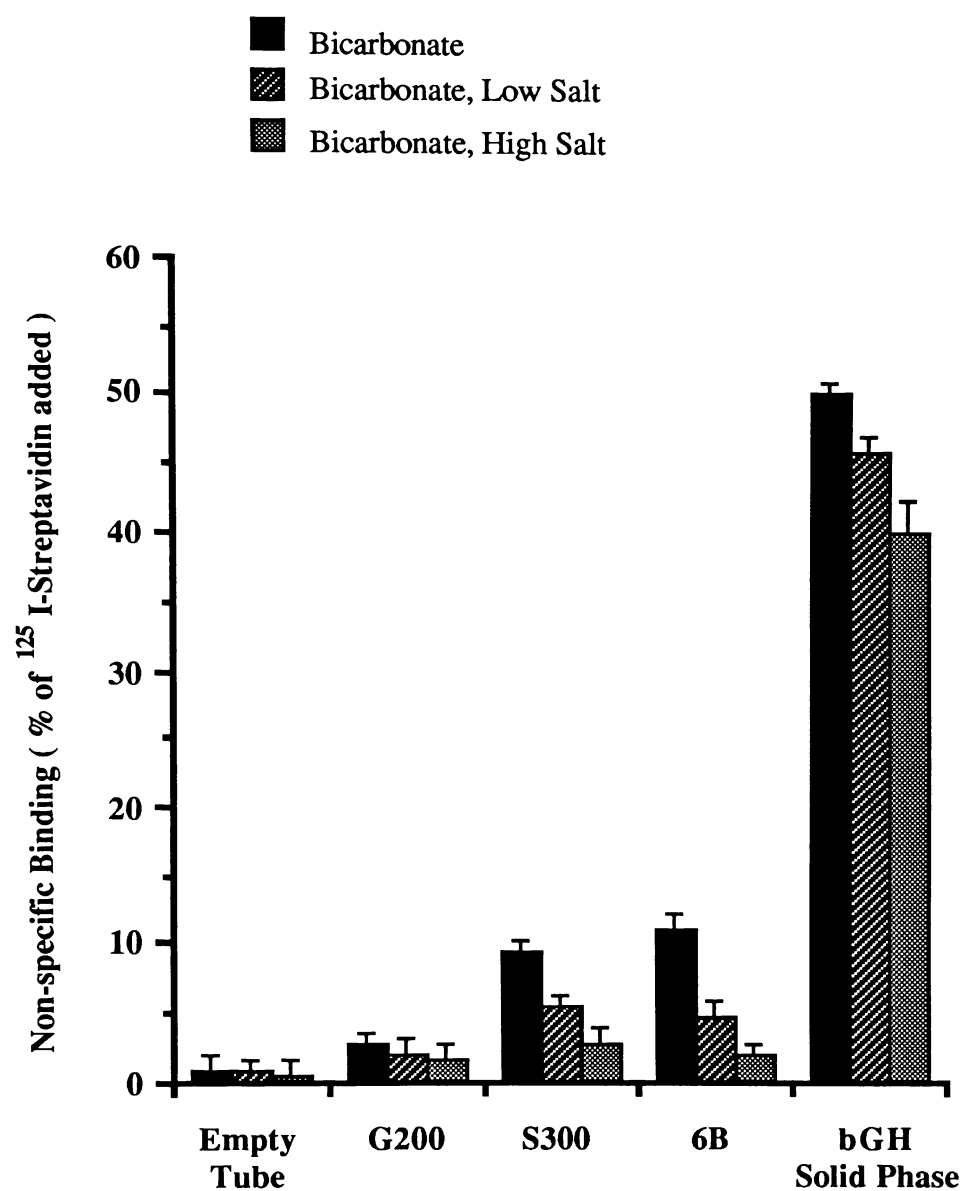


Figure 3.9 The effect of gel and salt concentrations in bicarbonate buffer on non-specific binding of 125 I-Streptavidin. ($\bar{X} \pm \text{SEM}$; n=3 replicates)

Incubation ~ Tubes were prepared for the binding assay as for the original incubation procedure. Primary antiserum was dispensed into all tubes except those which were controls for non-specific binding of the ^{125}I -streptavidin and second antibody. Following incubation and washing the second antibody was dispensed into tubes except the controls for non-specific binding of primary antibody and ^{125}I -streptavidin. Following a further incubation and washing, ^{125}I -streptavidin was added to all of the tubes. The contents of tubes with gel were mixed and washed before being centrifuged, the supernatant aspirated and the pellets gamma counted.

Results and Conclusion ~ The oGH solid phase and Sepharose 6B gel both show low non-specific binding of ^{125}I -streptavidin (Figure 3.10), approximately 3% of total counts, and no additional non-specific binding with the inclusion of the primary antibody. The control for non-specific binding of second antibody showed a significant increase with oGH solid phase to 16% of total counts, but little change for the 6B gel. The complete sequence for antibody detection of oGH showed total binding which was 43% of total counts, compared with non-specific binding to the 6B gel of 10% of total counts. Indications from the control for non-specific binding of second antibody were that up to 37% of the total binding observed with the complete incubation sequence and oGH solid phase could be non-specific. The incubation was repeated and yielded comparable results of non-specific binding of second antibody to the oGH solid phase, 30% of the total binding observed. Use of a biotinylated antibody raised in donkeys against rabbit serum (Amersham) also indicated high non-specific binding, 28% of total binding.

Because of the possibility that the biotinylated secondary antibody could bind non-specifically to proteinaceous or GH-like components of histological sections, no further investigations of the biotin-streptavidin system were undertaken.

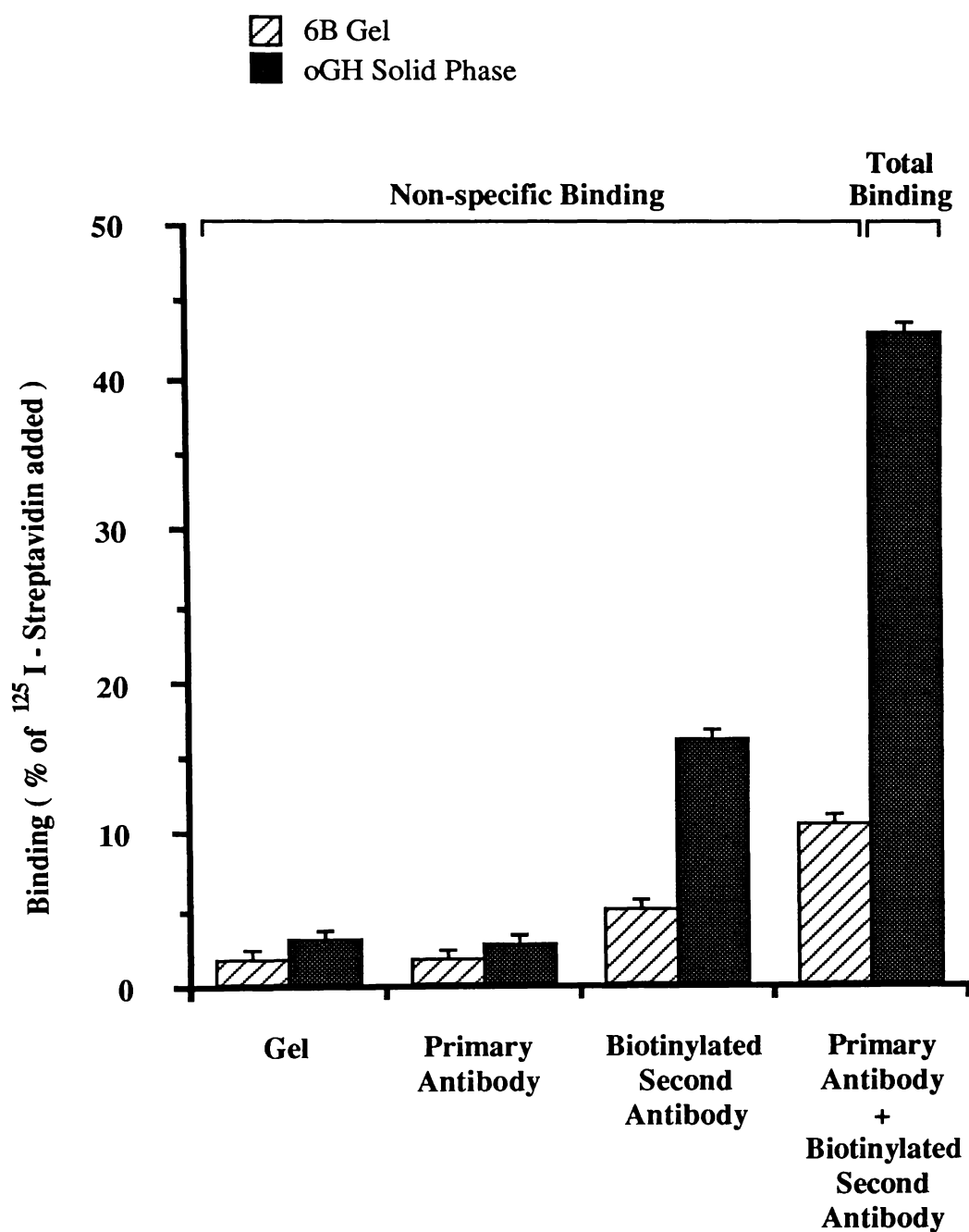


Figure 3.10 Sources of non-specific binding of ^{125}I -Streptavidin in sequential steps of the biotin-streptavidin assay
 ($\bar{X} \pm \text{SEM}$; n=3 replicates)

Binding of Iodinated Antibody to oGH Solid Phase and Liver Sections

The decision was made to utilise labelled ^{125}I -antibody directly, as experiments with labelling of a second antibody gave inaccurate results. A specific antibody against oGH and a non-specific control antibody were iodinated and used in this experiment. Both were tested initially in a solid phase binding assay, then by incubation with liver sections with gamma counting and protein assay.

Materials ~ PBSA was used as the antibody diluent and incubation buffer for the binding assay with solid phase. Ovine GH solid phase and Sepharose 6B gel were used in the assay. The non-specific antiserum was rabbit IgG, and both antibodies were used at an activity of 200,000 cpm/ml. The buffer and antibody diluent for incubation of liver sections was Tris-buffered saline (TBS; 0.05 M Tris HCl and 0.15 M sodium chloride). The specific and non-specific antisera were each used at activity levels of 150,000, 300,000 and 600,000 cpm/ml for the incubation of liver cryosections. Protein assay of sections proceeded as in the earlier experiment (p 86).

Animals and Tissue Preparation ~ The caudate lobe of liver was obtained at slaughter from a one year old wether. The liver was sliced in 5 mm strips and frozen in liquid nitrogen. The day before incubation, cryosections (8 μm) were cut at -16 to -18°C and thaw-mounted into tissue culture wells. These were returned to the freezing cabinet of the cryostat and then stored at -20°C until incubation.

Incubation with Ovine GH Solid Phase ~ Ovine GH solid phase and Sepharose 6B gel (1 ml) were prepared by washing with PBSA, briefly centrifuging and aspirating the PBSA (4 x 1 ml). The gels were resuspended in PBSA (2 ml), mixed and pipetted into tubes (200 μl). Duplicates of ^{125}I -antibody were added (100 μl) and aliquots of antibody placed in empty tubes for total counts. The tubes containing gel were mixed and incubated overnight at 4°C . PBSA was added (1 ml), the tubes centrifuged briefly, the

PBSA aspirated and the pellets gamma counted. The experiment was repeated with the same antibody solutions after they had been stored for ten days at 4°C.

Incubation of Liver Sections ~ This incubation protocol was based on that used for hormone incubation of cryosections but utilised TBS which is a more commonly used buffer for immunohistochemistry (Bourne, 1983). The sections were thawed and pre-incubated with TBS (1 ml, 10 min). The TBS was aspirated and ¹²⁵I-antibody placed over the sections (100 µl). Four sections were incubated for each combination of antibody and dilution, for two hours at room temperature. The incubation buffer was aspirated, the sections washed in TBS (2 x 5 min) and the TBS aspirated again. The sections were then precipitated, solubilised and assayed for protein. The incubation was repeated with freshly cut sections after the antibody solutions had been stored at 4°C for ten days.

Results and Conclusion ~ Data from the binding assay with gels (Figure 3.11) demonstrated good binding of the specific antiserum to oGH solid phase, 69% of total counts, with little non-specific binding to Sepharose 6B gel, 9% of total counts. The control antibody showed no greater binding to the oGH solid phase than to Sepharose 6B gel, 8% and 7% of total counts respectively. Lower, but reproducible binding was obtained with the antibodies after ten days in storage, 41% for the specific antiserum, and 2% to 4% for the control antibody.

The binding assay with liver sections (Figure 3.12) indicated no detection of specific binding of antibody at 150,000 cpm/ml, but some specificity of binding at both 300,000 and 600,000 cpm/ml. As a proportion of total binding, non-specific binding of the control antibody was much greater than in the solid phase assay, ranging from 23% to 78%. Again there was little deterioration of the antibodies after a ten day period.

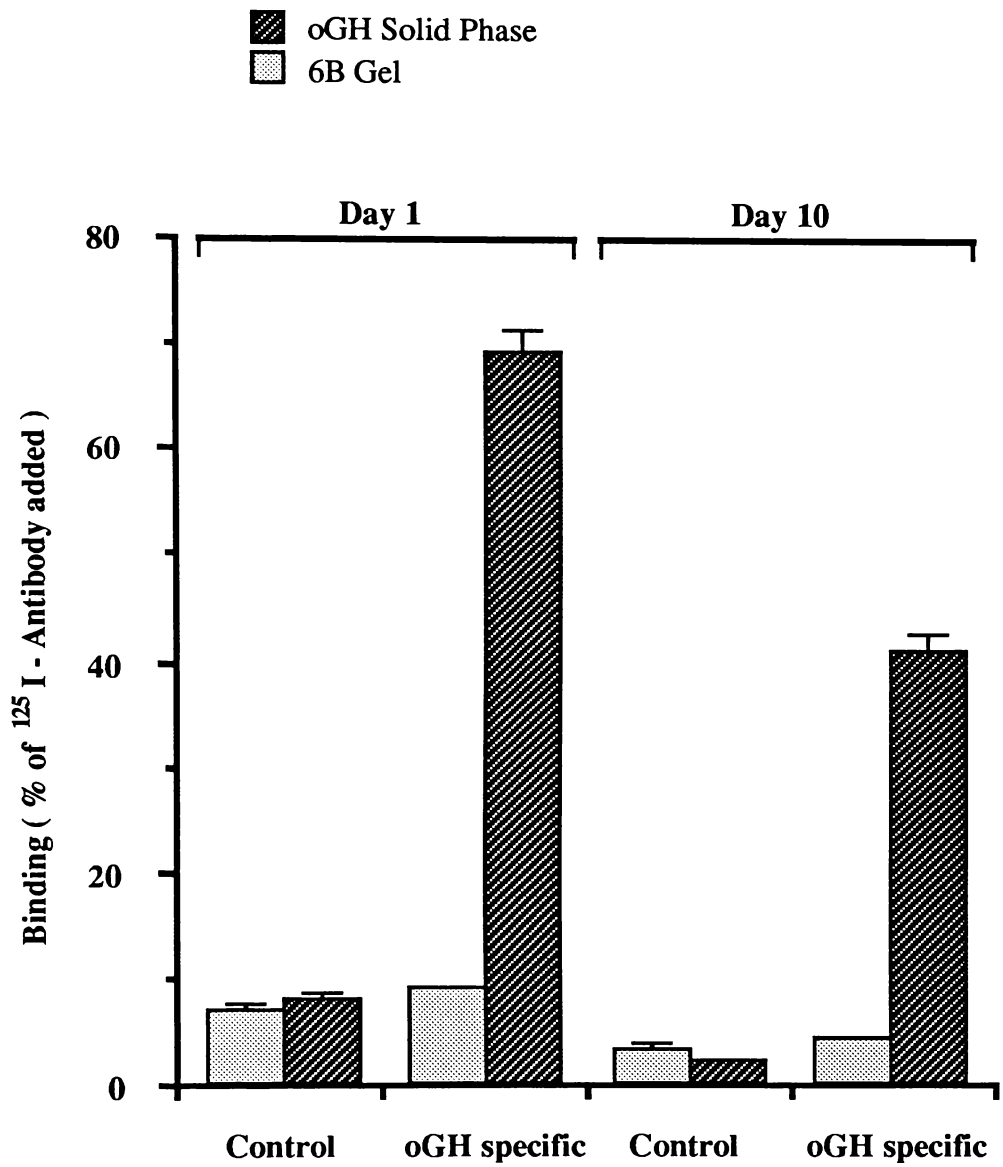


Figure 3.11 Binding of a specific antibody against GH and a control antibody, to oGH solid phase and 6B gel after 1 and 10 days of storage.
($\bar{X} \pm \text{SEM}$; n=2 replicates)

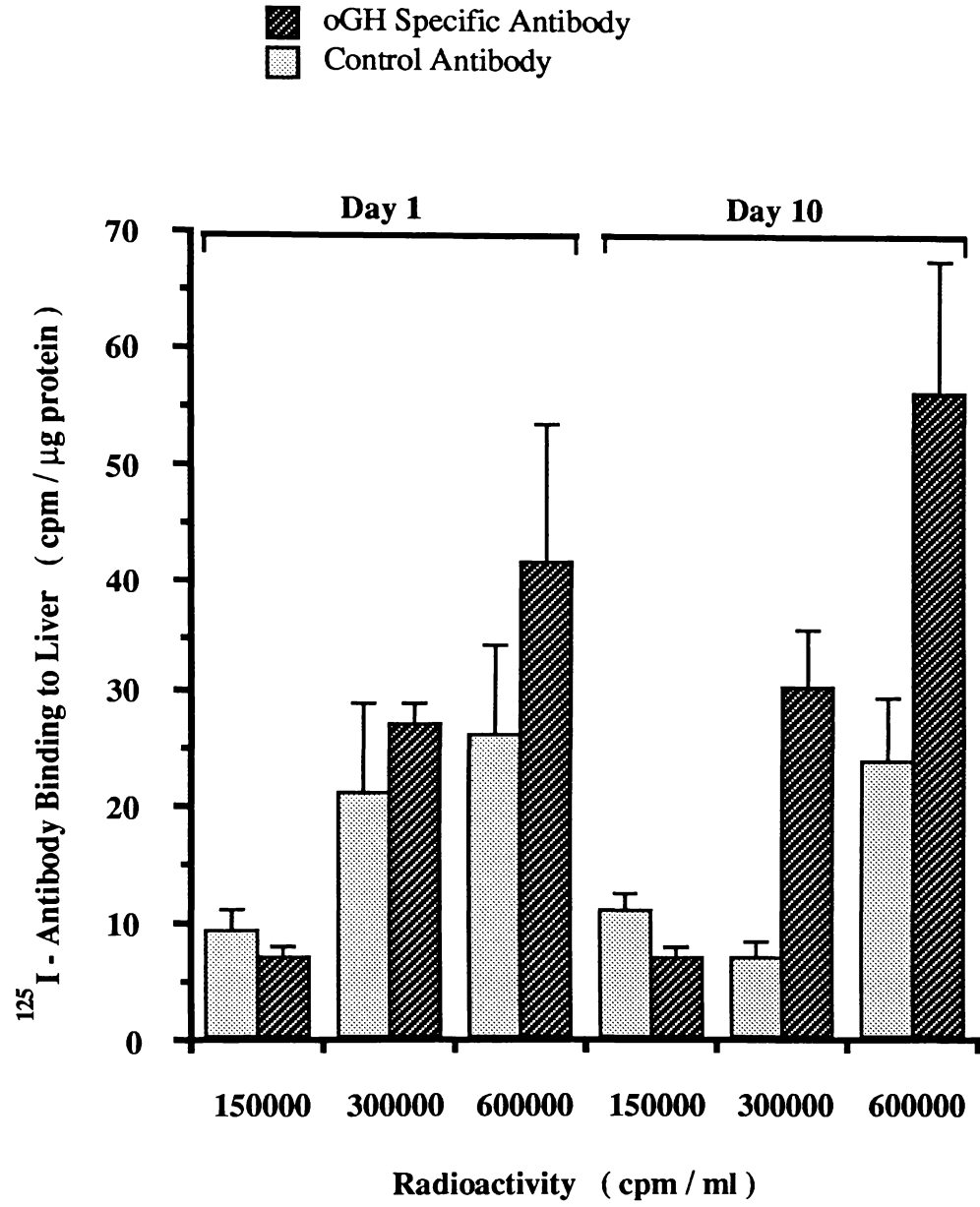


Figure 3.12 Binding of a specific antibody against GH and a control antibody, to cryosections of ovine liver after 1 and 10 days of storage.
($\bar{X} \pm \text{SEM}$; n=4 sections)

These assays demonstrated the specificity of ^{125}I -antibody binding, and a range of activities for the use of iodinated antibodies with cryosections. The antibody preparations were stable in incubation buffer over ten days at 4°C.

Fixation of Sections and Incubation Period

The effects of fixation of sections and incubation period on antibody binding were investigated. Specific and control antibodies for oGH were incubated with liver cryosections, and the sections then assayed for protein and gamma counted.

Materials ~ The incubation buffer was TBS, with the ^{125}I -antibodies used at an activity of 1,000,000 cpm/ml. Two fixatives were assessed; acetone, which is routinely used for immunodetection (Nairn, 1969; Grzanna, 1982), and Bouin's fluid. The Bouin's fluid is postulated to retain receptor integrity in membrane preparations (Salih *et al.*, 1979) so was possibly also appropriate for immunodetection in sections. Grzanna (1982) has also suggested that aldehyde based fixatives have little effect on the immunoreactivity of most antigens.

Incubation ~ Sections which were to be fixed were thawed and covered with acetone or Bouin's fluid (1 ml, 10 min). During this time the remaining sections were thawed. Fixatives were aspirated and all sections incubated with TBS (1 ml, 10 min). The TBS was aspirated and ^{125}I -antibody in TBS (150 μl) placed over the sections. The fixed sections and half the unfixed sections were incubated for two hours at room temperature, while the remaining unfixed sections were incubated overnight at 4°C. Following incubation, the buffer was aspirated, the sections washed with TBS and then precipitated and solubilised for gamma counting and protein assay.

Results and Conclusion ~ The data (Figure 3.13) showed no advantage of incubating sections overnight. Binding with the specific antibody was comparable but there was a 67% increase in binding of the control antibody with overnight incubation.

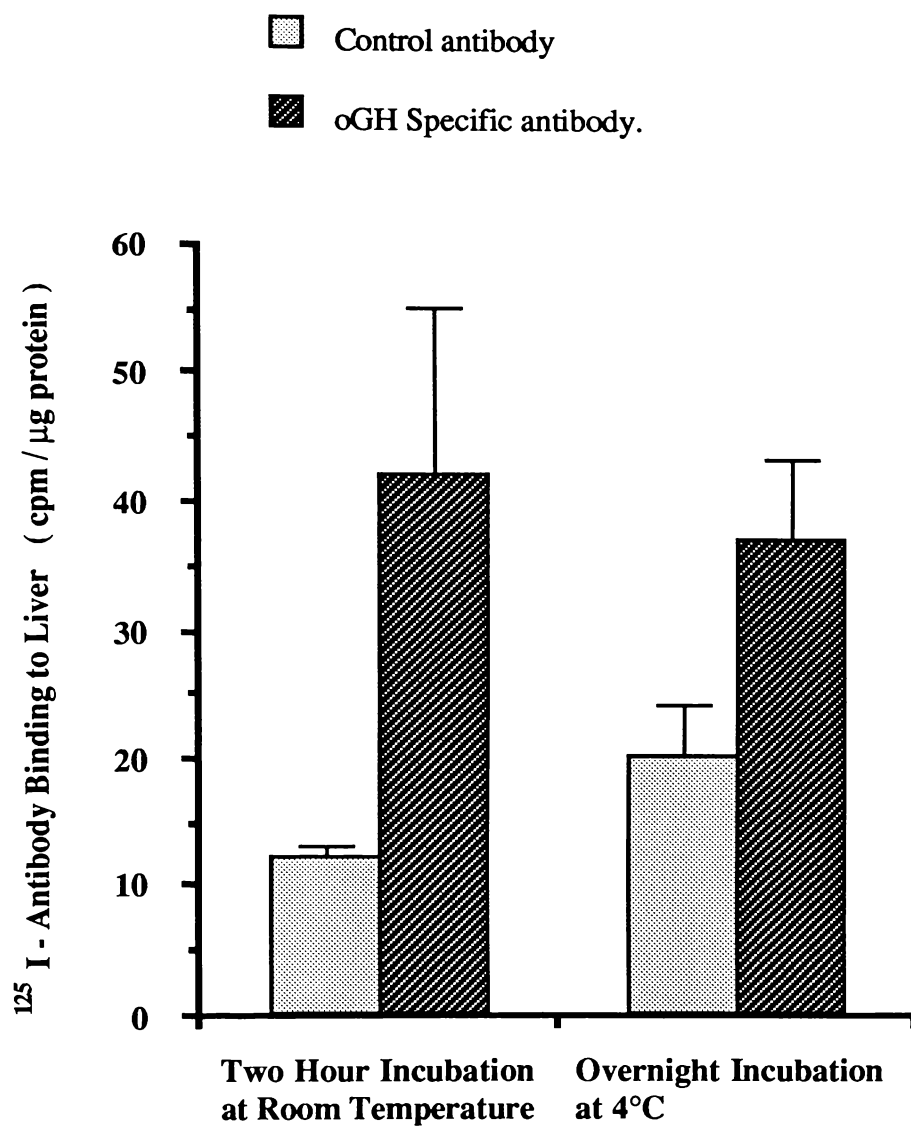


Figure 3.13 Effect of time and incubation temperature on binding to cryosections of ovine liver of a specific antibody against GH and a control antibody.
($\bar{X} \pm \text{SEM}$; n=4 sections)

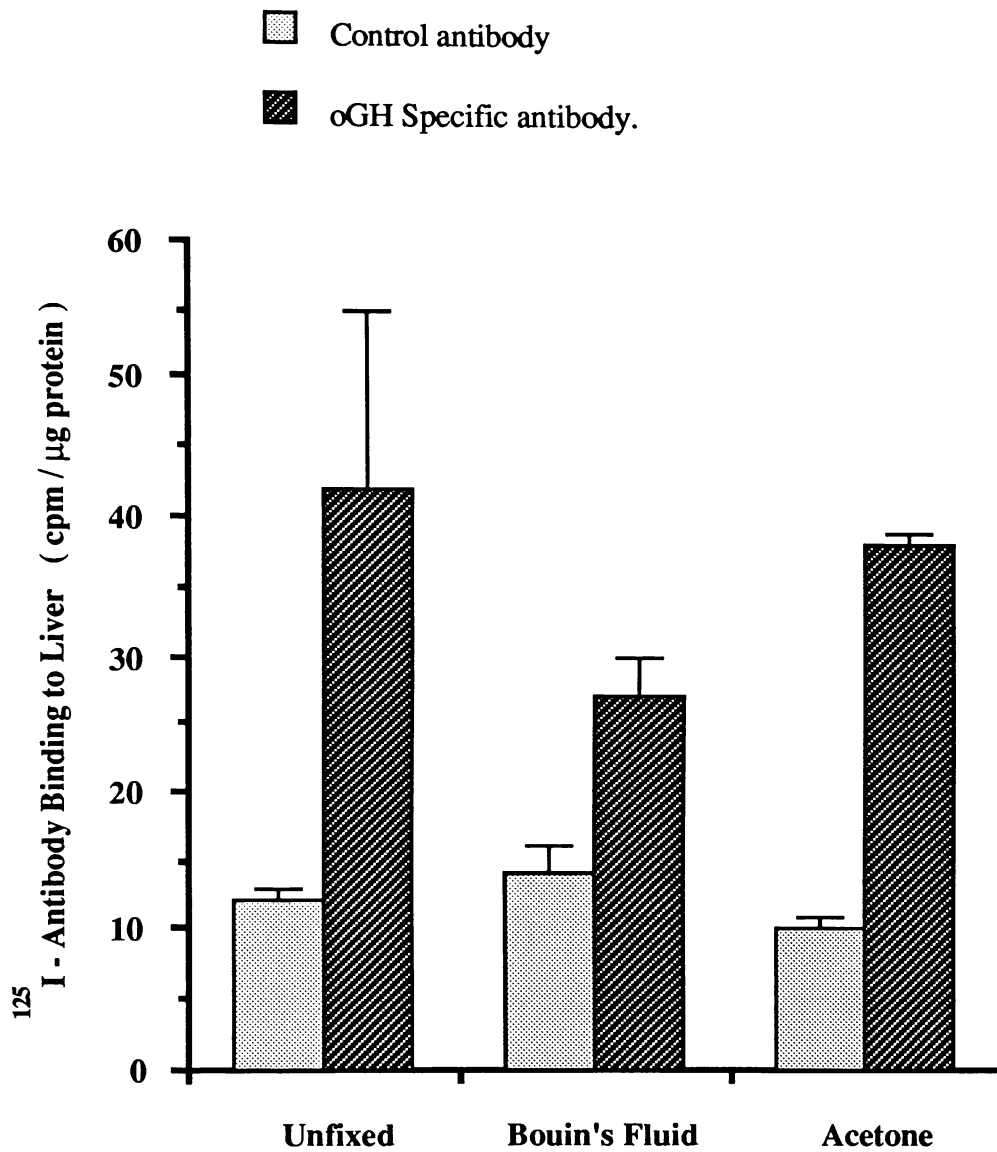


Figure 3.14 Effect of fixation on binding to cryosections of ovine liver of a specific antibody against GH and a control antibody.

($\bar{X} \pm \text{SEM}$; n=4 sections)

The fixed sections differed in their capacity for retaining antigenicity (Figure 3.14). Acetone fixation gave binding comparable with unfixed sections for both antibodies, but with binding of the specific antibody much less variable. Binding after fixation in Bouin's fluid was reduced for the specific antibody and marginally increased for the control antibody, resulting in under half the specific binding shown by the other treatments. There appeared to be no large change in antibody binding associated with an increase in activity to 1,000,000 cpm/ml when the data from the unfixed sections (Figure 3.14) were compared with data in Figure 3.12.

On the basis of these results, an incubation period of two hours with acetone-fixed sections was selected for autoradiography incubations. An activity of 400,000 cpm/ml was selected for the ^{125}I -antibody, in line with the level of radioactivity used in the hormone incubations. While measurement of specific binding at this level of activity may not be maximal, reduced levels of radioactivity are desirable out of consideration for safety, and to minimise the extent of non-specific binding.

Blocking of ^{125}I -Antibody with Antigen

With a quantitative study of binding, where the measurement of interest (specific binding) is always calculated by difference, it is of critical importance that the treatment *in vitro* of sections differs only by a single variable. With hormone incubations, the variable is the presence or absence of unlabelled hormone. It was considered that blocking of a labelled antibody with the antigenic hormone might be a more suitable control for non-specific binding than the use of a second labelled non-specific antibody such as rabbit IgG. This would avoid any problems with comparing two antibodies where there may be differences in purification, iodination efficiency, *etc.* This approach has been used in immunohistochemistry (Bloch, Brazeau, Ling *et al.*, 1983; Bennett-Clark and Joseph, 1986; Tolszczuk and Pelletier, 1988) and is recommended as an appropriate negative control (Dakopatts, 1987).

The aim of this experiment was to determine whether blocking of a high affinity antibody with its antigenic hormone would serve as a suitable control for non-specific binding in autoradiography incubations. Some sections were initially incubated with the antigenic hormone (unlabelled) before undergoing incubation with the ^{125}I -antibody, to increase the level of immunodetection.

Materials ~ The pre-incubation buffer was TBS. A blocking buffer of TBS with normal sheep serum (17%) was used to reduce non-specific binding of the ^{125}I -antibody to sections (Bourne, 1983). The incubation and post-incubation buffers were TBS with BSA (0.01%). The specific antibody was raised in sheep against recombinant hIGF1 at Ruakura Agricultural Centre. It was iodinated and used at an activity of 400,000 cpm/ml, with and without being blocked by preincubation with 1 $\mu\text{g/ml}$ hIGF1. The buffers for an initial incubation of some sections with unlabelled hIGF1 (1 $\mu\text{g/ml}$) were those used for hormone incubations (p 48).

Animal and Tissue Preparation ~ Cryosections were cut from a small number of muscle samples which had been collected and stored for the experiment described in Chapter 5. The 8 μm sections were cut at -16 to -18°C , thaw-mounted onto gelatinised slides and stored at -20°C until used within a week.

Incubation ~ Half the sections were incubated using the hormone incubation protocol but with unlabelled IGF1. Following the post-incubation washes they were shaken to remove most of the surface moisture and fixed in acetone (10 min). The remaining sections were thawed and also fixed in acetone (10 min). The acetone was aspirated and all of the sections pre-incubated with TBS (5 min). The TBS was aspirated and replaced with blocking buffer (20 min), which was then aspirated and replaced with incubation buffer containing ^{125}I -antibody (50 μl) with or without unlabelled IGF1. The sections were incubated at room temperature for two hours, and the incubation buffer aspirated. The sections were then washed in post-incubation buffer (2 x 5 min), distilled water

(5 min) and air-dried. Sections were placed against XAR film and exposed at room temperature for several days and the film images assessed following developing and fixing (p 51).

Results and Conclusion ~ Signal detection on the XAR autoradiographs from sections which were incubated directly with the antibody preparations was poor (not shown). There was no difference apparent between the sections incubated with ^{125}I -antibody and ^{125}I -antibody which was incubated earlier with IGF1. In contrast, the detection of hormone on sections which underwent an initial incubation with unlabelled IGF1 was significantly enhanced on those sections used to measure total binding. The difference between these and the corresponding sections incubated with pre-blocked antibody, representing nonspecific binding, was easily detectable on XAR film. The latter observation indicates that this protocol will specifically detect IGF1 in muscle cryosections, so it was utilised for autoradiography incubations with specific antibodies against IGF1 for the experiments described in Chapters 5 and 6.

CONCLUSION

The major difficulty encountered with developing the autoradiography protocols was the limited evidence on which to make technical decisions. To a certain extent protein assay, gamma counting and macroautoradiography were useful, but none of these techniques gave decisive data for making the final choice. The lengthy exposure periods for microautoradiography meant that this was not a suitable method for technique development, although it was the obvious choice for evaluating the effects of adjusting experimental procedure.

With the combination of techniques used in these experiments, the specific binding of radiolabelled hormone and antibody *in vitro* with cryosections has been demonstrated. The final incubation protocols (Chapter 2, p 48 and p 49) used for further work are not considered to be optimal, but are likely methods for successful autoradiographical assay

under a particular set of laboratory conditions. As the animal experiments progressed, further technical modifications to the protocols were made and these are outlined in the appropriate chapters. The production of autoradiographs remained as described in Chapter 2 (p 51) throughout the animal studies.

CHAPTER FOUR:

THE EFFECTS OF GRH AND TRH ON GH AND IGF1 IN PLASMA, GH AND IGF1 RECEPTORS IN LIVER, AND GROWTH

INTRODUCTION

Although GRH is the dominant hormonal stimulus of GH from the pituitary gland, a stimulatory effect of TRH on secretion of GH has been observed in numerous species (Harvey, 1990). In some studies a synergistic interaction between TRH and GRH on GH secretion has also been demonstrated (Taylor *et al.*, 1986; Welsh, Cuttler and Szabo, 1986; Lapierre *et al.*, 1987) and longer term TRH treatment of cattle can alter growth (McGuffey, Thomas and Convey, 1977).

Treatment of sheep with exogenous GRH clearly elevates plasma GH (Della-Fera *et al.*, 1986a; Barenton, Duclos, Diaz *et al.*, 1987) and several experiments have been performed to determine whether TRH acts similarly as a growth hormone releasing factor. Results to date have been inconclusive. Some studies have shown no effect of TRH on secretion of GH (Davis, 1975; Wrutniak *et al.*, 1987; Klindt, Davis and Ohlson, 1979; Muir and Wien, 1983), although Johnsson (1984) reported the onset of pituitary sensitivity of GH secretion to TRH in ewe lambs from six weeks old and marginal stimulation of GH secretion by TRH was described by Davis, Anfinson, Klindt *et al.*, (1977). Work *in vitro* with ovine pituitary cells has shown that TRH stimulates GH secretion (Takahara *et al.*, 1974; Hart *et al.*, 1988), but with much lower potency than GRH (Blanchard *et al.*, 1987).

There is one report of a study in new-born lambs where prolonged treatment with GRH has altered carcass composition in addition to elevating GH and IGF1 concentrations in plasma (Pastoureau, Charrier, Blanchard *et al.*, 1989). The changes in growth were similar to those observed with lambs which received exogenous GH (Butler-Hogg and Johnsson, 1987), the greatest effect being a reduction in carcass fat (Pastoureau *et al.*, 1989).

A primary target tissue for plasma GH is the liver. Hepatic IGF1 secretion is regulated by GH (Schwander *et al.*, 1983), and GH receptors may be up- or down-regulated by the prevailing GH status in plasma (Baxter *et al.*, 1982; Maiter *et al.*, 1988a). The number of Type 1 receptors is also regulated in number by prior exposure to GH and the IGFs (Corps and Brown, 1988; Lin *et al.*, 1988). Bass *et al.* (1991b) and Harding *et al.* (1987) have demonstrated increased hepatic binding of ^{125}I -GH in sheep following prolonged treatment with exogenous GH, but the effects of hormonally stimulated secretion of endogenous GH on hepatic GH receptors and, directly or indirectly, on hepatic Type 1 IGF receptors have not been reported for sheep.

The aims of this experiment were to determine whether long term treatment of prepubertal lambs with exogenous GRH and TRH would increase plasma concentrations of GH and IGF1 and, through stimulation of the GH-IGF axis, also promote growth. The receptor populations for GH and IGF1 in the liver were investigated as potential controlling variables by which changes in growth due to the experimental treatment might be mediated in peripheral tissues. T4 was measured as a positive control for stimulation of the thyroid hormone axis, ensuring the biological effectiveness of exogenous administration of TRH.

* The pellet composition was 60% lucerne, 30% barley, 5% linseed oil and 5% molasses.
Protein content was 17% and the metabolisable energy, 10 MJ/kg of dry matter.

MATERIALS AND METHODS

MATERIALS

Stock solutions of GRH (142 µg/ml) and TRH (66 µg/ml) were made at the beginning of treatment and frozen at -20°C in 20 ml aliquots. Both hormones were solubilised in acidified saline (pH≈2.5), the solution neutralised with sodium hydroxide and then made up to the required volume with additional saline. The aliquots were thawed overnight under refrigeration as required throughout treatment. GRH(1-29)NH₂ (RAA 19200) was kindly provided by Kabi Vitrum AB and TRH (catalogue number P-2161) was purchased from Sigma Chemical Company.

ANIMALS AND TREATMENT

Twenty five Suffolk x Coopworth wether lambs of 7-8 months in age were ranked on body weight and randomly allocated to five treatment groups (each n=5). There was a pre-trial range in body weight from 24 to 40 kg. The animals were housed indoors, penned individually and received a diet at 3% of body weight per day in pellets (NRM Feeds Limited)*. Water was available at all times and pellets were allocated at 08.45 daily. After a 21 day period for adjusting to these conditions, a group was slaughtered to provide pre-treatment data on body and carcass composition. At this time the other groups were started on the experimental schedule for 54 days of treatment. The daily treatments were as follows:

Control - injection of saline

GRH - injection of GRH (7.1 µg/kg) in saline

TRH - injection of TRH (3.3 µg/kg) in saline

GRH+TRH - injections of GRH (7.1 µg/kg) in saline and TRH (3.3 µg/kg) in saline

Dosages for GRH and TRH treatment approximated those successfully used by Brazeau *et al.* (1986) to stimulate GH secretion in cattle. The injection was administered as a subcutaneous bolus in the back of the neck each day at 08.30 before feeding. Each dose was delivered as one ml of solution per 20 kg of body weight. The GRH + TRH group

received their treatments as two separate injections. Body weights were recorded once a week, with hormone treatment and allocations of the pelleted feed adjusted accordingly for the following week.

BLOOD SAMPLING

Indwelling jugular vein catheters were inserted two days before treatment began and again on day 53 of treatment. Streptopen antibiotic (5 ml, Glaxo New Zealand) was administered by intramuscular injection to all animals on days 2, 3 and 4 of treatment to reduce the chance of infection associated with catheterisation. Blood samples were collected between 08.30 and 10.30 on the day before treatment began, between 06.30 and 20.30 on the first day of treatment and then between 06.30 and 08.30 on day 2. The same sampling regime for days 1 and 2 was used on day 54 of treatment and the following morning. Saline which was used during catheterisation and before sampling contained 25 000 IU heparin/l. Subsequently, saline containing 15 000 IU heparin/l was used to keep catheters patent during sampling. Blood samples (5 ml) were collected in heparinised tubes every twenty minutes for GH assay. Aliquots of heparinised plasmas were also taken every four hours for assay of IGF1 and T4. Blood samples were kept on ice until centrifuged under refrigeration and the plasmas then stored at -20°C until assayed.

PLASMA HORMONE ASSAYS

GH (Spencer *et al.*, 1991, p 56)

Duplicate aliquots of plasma and standards were prepared, rabbit anti-oGH antiserum added and then the tubes incubated for 24 hours. The following day, ¹²⁵I-GH was added with a second 24 hour incubation. On the final day, tubes were further incubated for two hours with goat anti-rabbit antiserum and normal rabbit serum in PEG. Additional PEG was added and the tubes centrifuged, the contents aspirated and the pellets gamma counted. The assay was completed within nine months of plasma collection.

IGF1 (Breier, 1987, p 58)

Plasma extraction to remove IGF binding proteins required dilution and incubation with an acid-ethanol mixture followed by neutralisation, centrifugation and assay of the supernatant. For assay, duplicate aliquots of standards and extracted plasmas were incubated with rabbit anti-hIGF1 antiserum for one hour. ^{125}I -IGF1 was added and the incubation continued for 24 hours. Subsequently, a mixture of sheep anti-rabbit antiserum and normal rabbit serum in PEG was added and the tubes further incubated for fifteen minutes. Tubes were then centrifuged, the contents aspirated and the pellets gamma counted. The IGF1 assay was completed within nine months of plasma collection.

T4 (Amersham International, p 59)

Plasma concentrations of free T4 were measured to provide a positive control for TRH treatment. Aliquots of plasma and standards were incubated with an iodinated derivative of T4 and a specific antibody against T4 for one hour. Tubes were then attached to a magnetic separation base, drained of supernatant and the magnetic pellets gamma counted.

AUTORADIOGRAPHY

At slaughter the caudate lobe of the liver was removed, weighed and partially sliced into 5 mm strips. These were frozen in liquid nitrogen and stored at -20°C . A small number of cryosections were cut and incubated using the protocol described for skeletal muscle in Chapter 2 (p 48). ^{125}I -GH (2.5 $\mu\text{g/ml}$) and ^{125}I -IGF1 (1.4 $\mu\text{g/ml}$) each at an activity of 400,000 cpm/ml were used as the radiolabelled hormones, with binding being competed for by the homologous hormones (1 $\mu\text{g/ml}$). Investigations in Chapter 3 revealed the presence of a brown pigmented material on microautoradiographs of liver (Plates 3.1 and 3.3). A number of treatments were applied to incubated cryosections in the current experiment during their processing as microautoradiographs to determine whether the material could be prevented from appearing. The treatments were:

- (1) Viewing of the cryosections without emulsion/stripping film.
- (2) Applying emulsion/stripping film to sections incubated with non-radioactive buffer.
- (3) Fixing of incubated sections with ethanol or acetone before applying emulsion.
- (4) Fixing of non-incubated sections with ethanol or acetone before applying emulsion.
- (5) Viewing of microautoradiographs before staining.
- (6) Reversal of stripping film on the sections so that gelatine layer rather than photographic emulsion was apposed to the section.
- (7) Placement of Formvar (Pelc, Coombes and Budd, 1961) between the sections and stripping film.
- (8) Speeding up of the drying process following application of stripping film to the sections.

GROWTH AND CARCASS COMPOSITION

The treatment groups were slaughtered in the research abattoir at Ruakura Agricultural Centre on the morning of day 57 following 56 days of hormone treatment. The animals were transported from their pens to the abattoir without being fed and were slaughtered on arrival. The following weights were recorded: final body weight, carcass, liver, thyroid glands and pituitary gland. The left side of each carcass was homogenised by mincing three times to provide a sample for analysis of carcass composition. The methodology for carcass analysis is based on that of Field *et al.* (1985), as outlined in Chapter 2 (p 59) and followed a procedure of freeze-drying to determine water content, extraction with petroleum ether for fat determination, heating of samples to ash to evaluate the contribution of bone and finally, the estimation of protein by difference.

STATISTICAL ANALYSIS

Blood concentrations of hormones were log transformed for statistical analysis. Comparisons were then made between treatment means using pooled SEDs. Other live

animal and slaughter measurements were analysed without log transformation. Gains in body weight were adjusted, using initial body weight as a covariate, to account for weight differences between treatment groups at the beginning of the experiment. Using ANOVA, all data were tested for main effects of the GRH and TRH treatments and their interactions.

RESULTS

PLASMA HORMONES

Plasma GH Concentrations

There was no significant effect of either hormone treatment on GH concentrations for day 1, although both GRH-treated groups had numerically greater levels of GH than the control and TRH-treated groups (Table 4.1). On day 54, a significant effect of GRH treatment on GH concentrations was apparent ($p \leq 0.001$), with GH almost five times higher than in the control and TRH groups. From day 1 to day 54 there was no significant change in GH concentrations within groups. Representative profiles of individuals, showing GH concentrations on the day before treatment began and for two hours immediately following treatment on day 1 and day 54, are presented in Figure 4.1. Clear elevation of GH was associated with GRH treatment, but there was little response to TRH or saline administration. The GH responses were variable between treatment days and animals, but in only three of the twenty profiles was there a failure of GRH treatment to elevate plasma GH immediately following injection (not shown). The pre-treatment profiles indicate that feeding alone was not associated with changes in plasma GH.

Plasma IGF1 Concentrations

On day 1 there was no effect of hormone treatment on IGF1 concentrations, but a significant effect of GRH treatment was apparent by day 54 (Figure 4.2, $p \leq 0.05$, $p \leq 0.01$).

Treatment	n	Day 1	Day 54	Pooled SED	Significance
Control	5	1.61	0.77	0.82	ns
GRH	5	2.18	3.10	0.82	ns
TRH	5	1.17	0.87	0.82	ns
GRH + TRH	5	2.39	4.62	0.82	ns
Pooled SED		0.57	0.57		
Significance (GRH)		ns	***		
Significance (TRH)		ns	ns		
Significance (GRH / TRH)		ns	ns		

Table 4.1 Plasma concentrations of GH (ng/ml) on day 1 and day 54 of animal treatment with saline, GRH (7.1 µg/kg/day) and/or TRH (3.3 µg/kg/day). The values are the group means. *** $p \leq 0.001$, ns not significant (ANOVA).

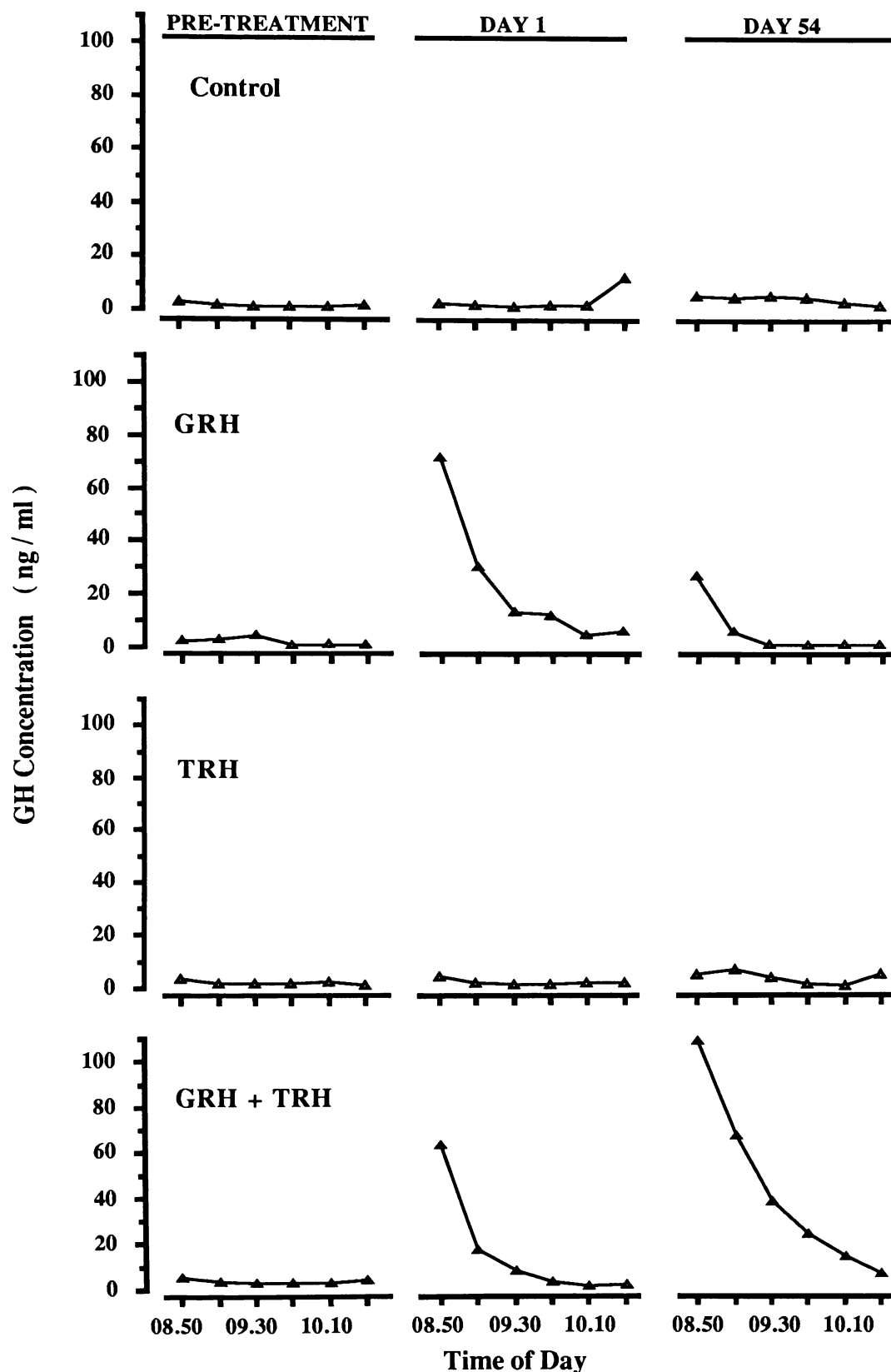


Figure 4.1 Plasma concentrations of GH (ng/ml) for the two hour period following feeding on the day before treatment began and following feeding and injection of animals with saline, GRH (7.1 μ g/kg/day) and/or TRH (3.3 μ g/kg/day) on day 1 and day 54 of treatment. Graphs show data from one animal in each group.

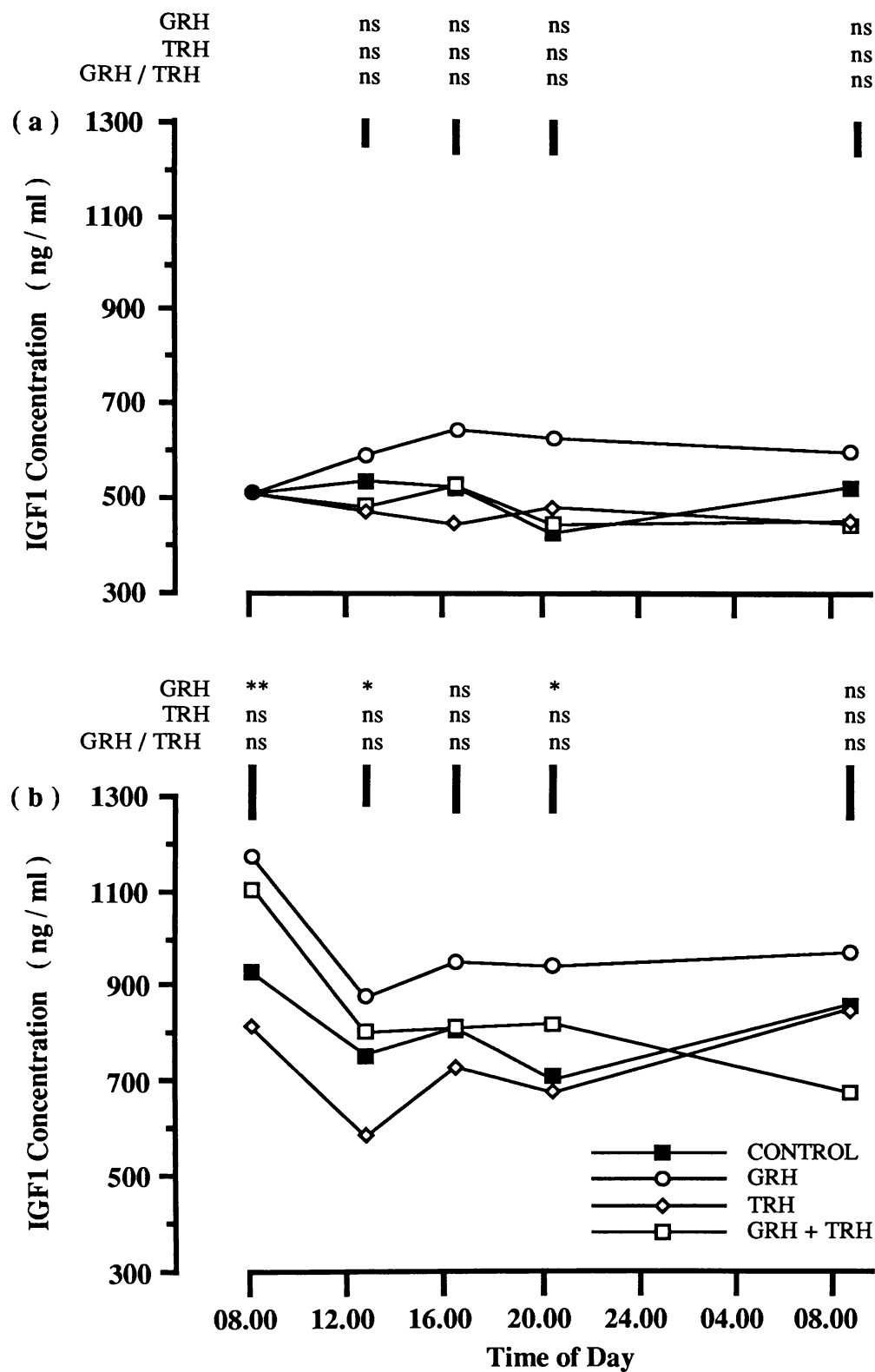


Figure 4.2 Plasma concentrations of IGF1 (ng/ml) following injection of animals with saline, GRH (7.1 µg/kg/day) and/or TRH (3.3 µg/kg/day) on day 1 (a) and day 54 (b) of treatment. Each data point is the group mean (n=5 per group) with the pooled SED shown above.

* $p \leq 0.05$, ** $p \leq 0.01$, ns not significant (ANOVA).

Additionally, all groups showed an increase in plasma concentrations of IGF1 between the first day of treatment and day 54 (grand mean: day 1, 515 ng/ml; day 54, 831 ng/ml; SED 52.7 ng/ml, $p \leq 0.001$).

Plasma T4 Concentrations

By four hours following injection of TRH or TRH + GRH on day 1, T4 concentrations were elevated (Figure 4.3, $p \leq 0.01$, $p \leq 0.001$). On day 54, not only was this trend absent but T4 concentrations tended to be lower in the TRH-treated animals than in the control and GRH-treated groups, although this was only significant by 24 hours following injection. There was no response of T4 to saline and GRH treatments. Only the GRH + TRH group showed a change in T4 concentrations from day 1 to day 54, decreasing with time (mean: day 1, 8.0 ng/ml; day 54, 6.6 ng/ml; SED 0.40 ng/ml, $p \leq 0.01$).

AUTORADIOGRAPHY

The brown pigment in microautoradiographs of liver was frequently found surrounding sinusoids and central veins. It was not present on sections which were not autoradiographs and possibly arose as a result of chemical reactions between endogenous precursors in the section and the photo-sensitive emulsion. The pigment could not be prevented from appearing or removed from autoradiographs by the various treatments of sections. Its presence, despite using numerous fixatives, suggests that it was not caused by fixation. It was present on autoradiographs before staining and whether or not the sections had been incubated, indicating that the incubation and staining procedures were not responsible. The placement of a gelatine barrier between the section and emulsion was not effective in preventing its appearance and the application of Formvar membrane did not result in autoradiographs of good quality. Likewise, the speed of drying the autoradiographs had no effect on the pigment.

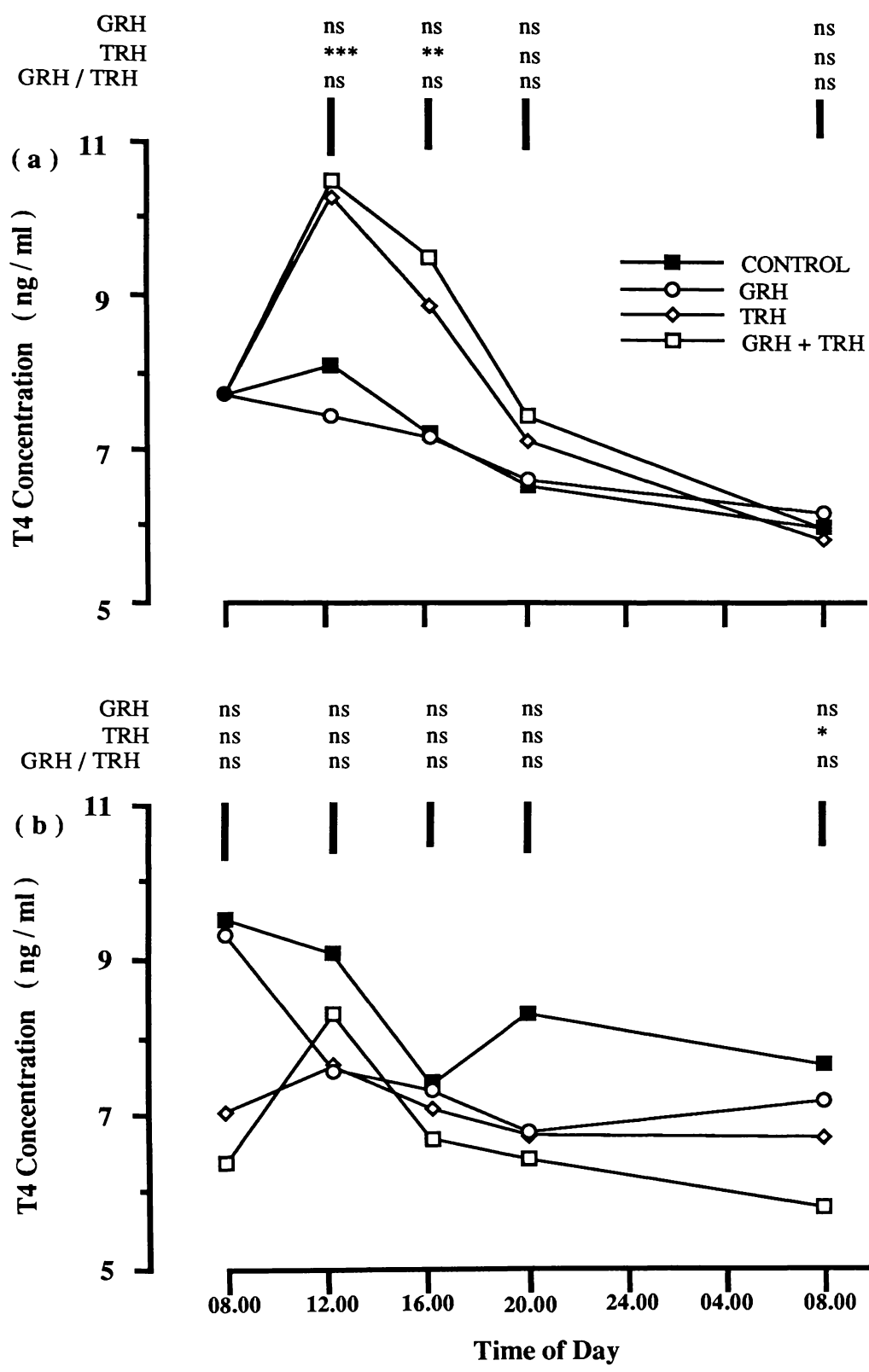


Figure 4.3 Plasma concentrations of T4 (ng/ml) following injection of animals with saline, GRH (7.1 μ g/kg/day) and/or TRH (3.3 μ g/kg/day) on day 1 (a) and day 54 (b) of treatment. Each data point is the group mean (n=5 per group) with the pooled SED shown above.

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns not significant (ANOVA).

Initially, counts were taken from sections, but the distinguishing of radiographic grains from the pigment was unreliable, so the autoradiography incubations which were planned to measure GH and IGF1 receptors in the liver were abandoned.

GROWTH AND CARCASS COMPOSITION

Weight gains did not differ between the main effects of treatments until the final week when a positive overall effect of TRH on weight was apparent (Figure 4.4, $p \leq 0.05$). This appears to have been a consequence of declining weight gain in the control group and a continued greater gain of weight in the GRH + TRH group. For the final three weeks of treatment there was also an interactive effect of GRH and TRH treatment ($p \leq 0.05$). The weights measured at slaughter (Table 4.2) are presented as differences from the means of the pre-treatment group. Measurable effects of treatment with GRH or TRH were limited to an increase in size of the pituitary glands, which was associated with GRH treatment ($p \leq 0.05$). The data for carcass composition (Table 4.3) are also presented as differences from the means of the pre-treatment group. These data showed no effects of the experimental treatment.

DISCUSSION

EFFECTS OF GRH AND TRH ON PLASMA HORMONES

Subcutaneous administration of GRH was effective in raising GH concentrations to above unstimulated levels (Table 4.1), apparently without the consequence of pituitary refractoriness with several weeks of daily treatment. This contrasts with the study of Della-Fera *et al.* (1986a) who observed significant declines in the response of plasma GH with daily injections of GRH over a few days. A temporary attenuation of GH in response to GRH may have occurred during the current experiment but if so, responsiveness was restored before the conclusion of treatment. The enlargement of the pituitary gland in the GRH-treated sheep (Table 4.2) would support this.

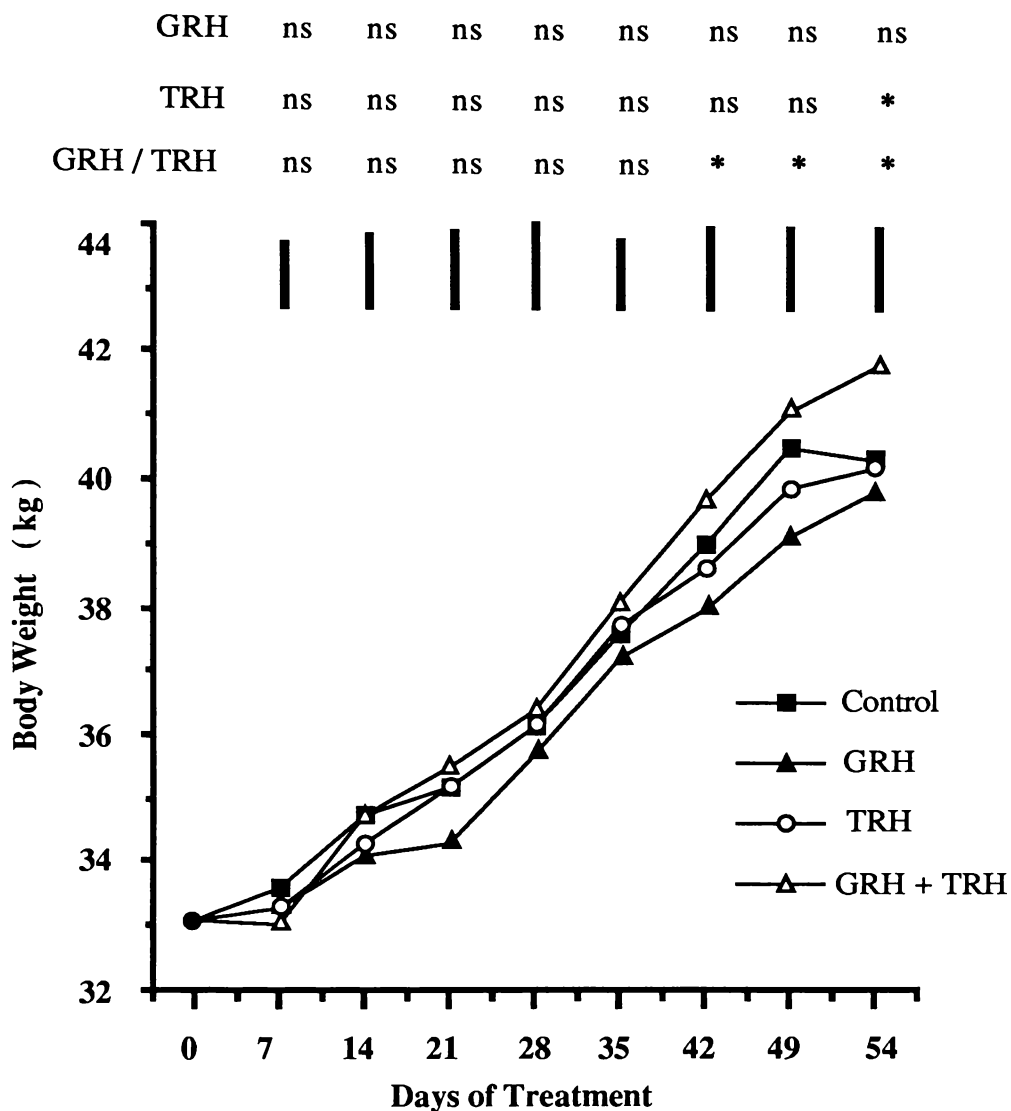


Figure 4.4 Body weight (kg) of animals throughout period of treatment with saline, GRH ($7.1 \mu\text{g/kg/day}$) and/or TRH ($3.3 \mu\text{g/kg/day}$). Each data point is the group mean, after co-variate adjustment for differences in body weight at the beginning of the experiment, with the pooled SED shown above ($n = 5$ per group). * $p \leq 0.05$, ns not significant (ANOVA).

Treatment	n	Final Liveweight (kg)	Carcass (kg)	Liver (g)	Pituitary (g)	Thyroid (g)
Pretreatment Group	5	30.6	16.1	494	0.49	2.34
Control	5	+ 10.8	+ 4.6	+ 253	0.00	+ 0.91
GRH	5	+ 9.7	+ 3.7	+ 319	+ 0.14	+ 2.42
TRH	5	+ 7.6	+ 3.3	+ 91	+ 0.05	+ 1.09
GRH + TRH	5	+ 11.6	+ 4.7	+ 284	+ 0.13	+ 2.89
Pooled SED		2.23	1.28	83.6	0.051	0.787
Significance (GRH)		ns	ns	ns	*	ns
Significance (TRH)		ns	ns	ns	ns	ns
Significance (GRH / TRH)		ns	ns	ns	ns	ns

Table 4.2 Changes in weight of body components following 54 days treatment of animal with saline, GRH (7.1 µg/kg/day) and/or TRH (3.3 µg/kg/day). The values are weight differences from means for a group of untreated animals which were slaughtered at the beginning of the experiment. * $p \leq 0.05$, ns not significant (ANOVA).

Treatment	n	Water (kg)	Fat (kg)	Protein (kg)
Pretreatment Group	5	8.1	4.7	2.7
Control	5	+ 2.4	+ 1.5	+ 0.5
GRH	5	+ 2.2	+ 0.9	+ 0.3
TRH	5	+ 1.6	+ 1.4	+ 0.1
GRH + TRH	5	+ 2.7	+ 1.2	+ 0.4
Pooled SED		0.59	0.62	0.23
Significance (GRH)		ns	ns	ns
Significance (TRH)		ns	ns	ns
Significance (GRH / TRH)		ns	ns	ns

Table 4.3 Carcass composition following 54 days of animal treatment with saline, GRH (7.1 µg/kg/day) and/or TRH (3.3 µg/kg/day). The values are weight differences from the means for a group of untreated animals which were slaughtered at the beginning of the experiment. ns not significant (ANOVA).

In previous studies with sheep, GRH administration has mostly been by intravenous injection, with stimulation of GH obtained at doses of up to 10 µg/kg GRH (Barenton *et al.*, 1987; Kensinger, McMunn, Stover *et al.*, 1987; Wrutniak *et al.*, 1987). A comparison has been made between GRH administration by intravenous or subcutaneous injection, with the conclusion that subcutaneous administration was effective but higher doses were required and the effect on GH secretion was less prolonged (Della-Fera *et al.*, 1986b). Similarly, Kensinger *et al.* (1987) have determined that multiple injections of GRH are a more effective stimulant of GH than a continuous infusion.

Three of the twenty GH profiles for individual animals did not show a post-injection rise in GH concentrations immediately following GRH treatment. Depletion of GH or refractoriness of pituitary somatotropes after repeated stimulation with GRH *in vitro* has been postulated to occur (Vance *et al.*, 1986; Richardson and Twente, 1988). Whether a similar phenomenon *in vivo*, where injection may have closely followed an endogenous peak of GRH, is responsible in these three non-responding cases cannot be determined. An alternative explanation is that the injections on these occasions were delivered intradermally rather than subcutaneously, possibly retarding clearance to plasma. It has also been found that sheep are variable in their response to GRH (Kensinger *et al.*, 1987) and those which are most sensitive to stimulation of GH by GRH have an endogenous rhythm of high amplitude pulses of GH (Barenton *et al.*, 1987). Individual sensitivity is unlikely to be the explanation for the profiles which showed no GH response, as this occurred once for each of three animals, all of which showed a post-injection peak of GH on the other day of sampling.

GRH treatment resulted in a transient elevation of IGF1 concentrations on day 54 of treatment in the present study (Figure 4.2). Others have also found that plasma IGF1 concentrations increase in association with GRH-stimulated increases of plasma GH in sheep (Della-Fera *et al.*, 1986a). This has not been observed with shorter term GRH treatment of younger lambs (Della-Fera *et al.*, 1986a) and calves (Coxam *et al.*, 1987)

and may arise as a consequence of post-natal maturation of the GH-IGF axis (Gluckman and Butler, 1983).

The effectiveness of subcutaneous injection of TRH is indicated by the peak in T4 concentrations after treatment on day 1 (Figure 4.3). Wrutniak *et al.* (1987) have previously established that TRH administration in sheep is equally as effective by subcutaneous or intravenous routes, with little effect of dosage on the T4 response other than prolonging of the T4 peak. In the present study there is a suggestion of insensitivity of the thyroid gland to stimulation by TRH on day 54. Pre-injection concentrations of T4 tended to be less in TRH-treated animals, increasing to within the range shown by other groups following injection, and then declining again. Earlier data (Muir and Wien, 1983) showed elevated T4 concentrations in lambs following eight weeks of TRH treatment by continuous infusion. Enlargement of the thyroid gland, as observed by Muir and Wien (1983), was not significant in the present experiment, although the glands did appear larger (Table 4.2). Possibly the mode of TRH administration, chronic versus acute, or the greater dosage used by Muir and Wein may account for this contradictory finding.

The absence of an effect of TRH on GH concentrations (Table 4.1) supports the data of earlier studies with sheep (Davis, 1975; Klindt *et al.*, 1979; Wrutniak *et al.*, 1987). Using wethers of 20 to 30 kg in body weight, Davis, Hill, Ohlson *et al.* (1976) found no increase in plasma GH with chronic TRH treatment for two weeks. They reported no change in plasma GH with twice daily injections of TRH, except at a pharmacological dose (1 mg/ml). However, a marginal increase in plasma GH eight hours after TRH injection has been observed (Davis *et al.*, 1977b). In a study with ewe lambs, Johnsson (1984) determined that the animals acquired pituitary sensitivity to TRH at six to eight weeks of age and became insensitive again at approximately 26 weeks. He also observed that the magnitude of GH secretion stimulated by TRH was highly variable between individuals (Johnsson, 1984). Evidence obtained *in vitro* (Takahara *et al.*, 1974; Blanchard *et al.*, 1987) indicates that TRH is a positive stimulus of GH from the ovine

pituitary, but this appears difficult to demonstrate *in vivo*. Because of variability in the GH response to TRH, the size of groups in the current experiment may have precluded observing small but significant treatment effects, particularly considering that the age of the animals may not have been optimal for such a demonstration.

There were no statistically significant interactions between the effects of GRH and TRH treatments on the plasma hormones which were measured. However, GH concentrations on day 54 in particular suggest that GRH and TRH show synergism in stimulating GH secretion. GH concentrations were 50% greater in the GRH + TRH group than in the GRH group. This observation is supported by a finding *in vitro* with ovine pituitary cells (Hart *et al.*, 1988) but the synergism was not strong enough to cause the elevation of plasma IGF1.

EFFECTS OF GRH AND TRH ON GROWTH AND CARCASS COMPOSITION

The treatments did not affect growth (Figure 4.4, Table 4.2, Table 4.3). As the growth effects of GRH are considered to be mediated by GH and/or IGFs, the observed stimulation of endogenous GH and IGF1 might have been expected to result in changes in body weight and carcass composition comparable with treatment with exogenous GH. Treatment of lambs with exogenous GH produces little change in body weight (Muir *et al.*, 1983; Rosemberg *et al.*, 1989) although more recently Bass *et al.* (1991a) have demonstrated a dose-related body weight response in sheep receiving daily injections of GH. Data from the current experiment suggest that the stimulation of GH and IGF1 by GRH was insufficient to effect a measurable increase in body weight gain. A predominant consequence of GH treatment of lambs is a reduction of carcass fat (Wagner and Veenhuizen, 1978; Johnsson, Hathorn, Wilde *et al.*, 1987). There was not a significant effect of GRH treatment on carcass fat in this study, further supporting the idea that stimulation of the GH-IGF axis was insufficient to have effects on the carcass at this level of GRH administration. Treatment with greater concentrations of GRH might have effected changes in growth through the GH-IGF axis. Pastoureau *et al.* (1989)

altered growth in newborn lambs with approximately double the daily dose of GRH which lambs in the current experiment received.

Limited observations of TRH effectiveness as an ovine growth promotant have been made, with the changes in growth being equivocal (Davis *et al.*, 1976; Davis *et al.*, 1977b; Muir and Wien, 1983). The hypothesis that TRH is a growth promotant is not supported by various data from the current experiment. The same dose of exogenous TRH used in cattle is a potent stimulus of GH (Brazeau *et al.*, 1986), with the likelihood that growth could be altered through the GH-IGF axis following long-term treatment with TRH. In lambs, pituitary sensitivity to TRH is variable between individuals and limited to between the first and sixth months of life (Johnsson, 1984), after which time a pharmacological concentration of TRH is required to elevate GH concentrations (Davis *et al.*, 1976). Together, these factors make it highly unlikely that TRH would be an effective growth promotant in sheep.

There was limited evidence of synergism between GRH and TRH treatments on later gains in body weight during the experiment (Figure 4.4). The hormone data in this experiment suggest that this has not resulted via stimulation of the thyroid hormones or IGF1 concentrations, but Rosemberg *et al.* (1989) reported greater weights of muscle and protein with GH and T4 treatment compared with no effect of either treatment alone. In both studies the effects were small, in line with the marginal synergism suggested by secretory studies of the pituitary *in vitro* (Blanchard *et al.*, 1987).

CONCLUSION

After numerous trials and treatments it is concluded that microautoradiography is not a suitable method to use for receptor analysis of ovine liver. A further treatment which may be explored is the removal of pigment following production of the autoradiographs, but the concurrent removal of radiographic grains would be difficult to monitor.

The remaining experiments in this thesis utilised skeletal muscle for analysis of GH and IGF receptors.

In this experiment, administration of exogenous GRH failed to influence animal growth and carcass composition despite increasing GH and IGF1 concentrations in plasma. Subcutaneous administration of GRH was successful but probably did not stimulate the secretion of GH from the pituitary sufficiently to effect the changes in carcass composition which are usually associated with elevated GH concentrations in well nourished sheep *i.e.* reduced carcass fat. The potential for GH stimulation by GRH may also have been influenced by other experimental parameters. Changes in growth might have been achieved with the use of multiple injections of GRH throughout the day (Kensinger *et al.*, 1987) or an intravenous route of administration (Della-Fera *et al.*, 1986b).

There was no evidence that TRH stimulated GH secretion, increased growth or changed carcass composition. This is not likely to have been caused by ineffective administration of the hormone as effects of TRH on thyroid secretion of T4 were apparent on both sampling days. There is a real probability that the age of the animals in the current experiment was not optimal for the demonstration of pituitary effects of TRH on GH secretion. It is also possible that the sex of the animal may alter the effectiveness of TRH as a GH stimulant and growth promotant in sheep, the positive results of Johnsson (1984) having been obtained with ewe lambs. Synergism of GRH and TRH treatments was limited to the observation of non-significant effects on plasma GH and small changes in growth.

In conclusion, unlike numerous other species (Harvey, 1990), sheep do not show a great sensitivity of pituitary GH secretion to TRH, alone or synergistically with GRH. Work *in vitro* suggests that the ovine pituitary gland is capable of secreting GH in response to TRH so, as postulated by Johnsson (1984), there may be development of inhibitory

controls *in vivo* in the growing lamb which cannot be countered with the use of exogenous TRH.

CHAPTER FIVE:

THE EFFECTS OF NUTRITION AND GH ON GH AND IGF1 IN PLASMA, GH AND IGF1 RECEPTORS IN SKELETAL MUSCLE, AND GROWTH

INTRODUCTION

In domestic ruminants there have been numerous studies of the effects of diet on weight gain and carcass composition (Jagusch, Rattray, Petch *et al.*, 1980; Seideman and Crouse, 1986) which, more recently, have included measurements of plasma concentrations of GH and IGF1 (Waghorn, 1986; Bass *et al.*, 1987) to investigate the hormonal mechanism of growth under different nutritional regimes. Dietary components which affect plasma GH and IGF1 include the content of protein and trace elements (Kung *et al.*, 1984; Emery *et al.*, 1986). With restricted nutrition, GH concentrations in cattle are elevated and IGF1 concentrations are depressed (Breier *et al.*, 1988b; Elsasser *et al.*, 1989).

GH is a central hormone in the control of post-natal growth in normal animals. Hypophysectomy results in retarded growth which can largely be reversed by treatment with GH (Young *et al.*, 1989). With the advent of large quantities of recombinantly produced GH, the effects of exogenous administration to domestic species is also well documented (Muir *et al.*, 1983; Johnsson *et al.*, 1985), with a prominent effect being reduced levels of fat and elevated protein in the carcass (Pell and Bates, 1987).

GH can alter growth independently of nutritional constraint, but how the regulatory control of growth by GH may be further influenced by nutrition is unknown. The mechanisms by which growth is achieved in somatic tissues remains to be defined and gaining an understanding of them necessitates investigating hormone receptors in target tissues in addition to the secretion of hormones into plasma. The aim of this experiment was to investigate the regulation of growth by nutrition and GH through the GH-IGF axis, and to determine the role which might be played by GH and Type 1 IGF receptors in skeletal muscle.

MATERIALS AND METHODS

MATERIALS

Lyophilised GH (recombinantly derived bovine GH, BST) was reconstituted as a stock solution of 10 mg/ml with distilled water (100 ml). It was refrigerated at 4°C and used over a period of 7-10 days. The BST was kindly donated by American Cyanamid Company.

A complete pelleted diet was purchased from NRM Feeds Limited.

ANIMALS AND TREATMENT

Twenty five Suffolk x Coopworth wether lambs of 7-8 months in age were ranked on body weight and randomly allocated to five treatment groups (each n=5). There was a pre-trial range in body weights from 24 to 31 kg. The animals were housed indoors, penned individually and received a diet at 3% of body weight per day in pellets. Water was available *ad libitum* at all times and pellets were allocated at 09.15 daily. After a fourteen day period of adjustment to these conditions, a group was slaughtered to provide pre-treatment carcass data. The other groups remained under the pre-treatment conditions for a further week and then started on the experimental schedule for 42 days of treatment. The treatments were as follows:

High Nutrition - 3% of body weight/day in pellets. Saline injected at 0.5 ml per day.

Low Nutrition - 1.25% of body weight/day in pellets. Saline injected at 0.5 ml per day.

High Nutrition + BST - 3% of body weight/day in pellets. BST injected at 0.25 mg per kg per day.

Low Nutrition + BST - 1.25% of body weight/day in pellets. BST injected at 0.25 mg per kg per day.

Injections were delivered as a single, subcutaneous bolus (0.5 ml per 20 kg of body weight) in the back of the neck at 09.00 daily. Body weights were recorded weekly, with BST doses and pellet allocations adjusted accordingly for the following week.

When necessary throughout the trial, animals which were noted as refusing substantial amounts of feed for two or more days received a course of Streptopen antibiotic. Three daily injections of 5 ml were administered to four animals. Four animals also received grass mixed with their pellets on up to three occasions to improve consumption. At slaughter, one animal which had received both antibiotic and the supplement of grass was diagnosed as suffering from pleurisy, so all data collected from this animal were excluded from analysis.

BLOOD SAMPLING

Indwelling catheters were inserted into a jugular vein on day 39 of treatment and five ml samples collected at twenty minute intervals from 08.00 to 21.00 on day 42. Catheters were flushed with saline which initially contained 25 000 IU heparin/l, then 15 000 IU heparin/l throughout sampling to keep the catheters patent. Blood samples were collected into heparinised tubes and kept on ice. Plasma was separated by centrifugation and stored at -20°C until assayed. The twenty minute samples were assayed for GH and aliquots of plasma were taken every four hours for IGF1 assay.

PLASMA HORMONE ASSAYS

GH (Breier, 1987, p 57)

Aliquots of sample and standards were incubated with an antibody which had been raised in rabbits against oGH. ^{125}I -GH was added and, following a further incubation, a mixture of sheep anti-rabbit antiserum, normal rabbit serum and PEG also added. Additional PEG was added, the tubes were centrifuged, the supernatant aspirated and the pellets gamma counted. The assay was completed within one month of sample collection.

IGF1 (Breier, 1987, p 58)

Aliquots of standards and extracted plasmas were incubated with rabbit anti-hIGF1 antiserum. ^{125}I -IGF1 was added and, following a period of further incubation, a mixture of sheep anti-rabbit antiserum, normal rabbit serum and PEG also added. Tubes were then centrifuged, the supernatant aspirated and the pellets gamma counted. This assay was completed within eleven months of sample collection.

AUTORADIOGRAPHY

At slaughter, pieces of the *M. semi-membranosus* of the hind limb were removed and frozen in liquid nitrogen. The samples were stored at -20°C until cryosections were cut at -16°C . Sections of $8\text{ }\mu\text{m}$ thickness were cut, thaw-mounted onto gelatinised microscope slides at room temperature and immediately replaced in the freezing cabinet of the cryostat. Sections remained frozen until incubation, always within one week of being cut. The incubation protocols for use with iodinated hormone and antibody are outlined in Chapter 2 (p 48 and 49). For each animal and each incubation, three pairs of cryosections were used. Within each pair, one slide was randomly allocated for the assessment of total binding, the other being used to assess non-specific binding (Figure 2.1).

Hormone incubations were performed using ^{125}I -GH (2.5 ng/ml) $\pm$ GH (1 $\mu\text{g/ml}$), ^{125}I -IGF1 (1.4 ng/ml) $\pm$ IGF1 (1 $\mu\text{g/ml}$), ^{125}I -IGF1 $\pm$ insulin (10 ng/ml) and ^{125}I -IGF1 $\pm$ insulin (10 $\mu\text{g/ml}$). The radiolabelled hormones were used within fifteen days of iodination at an activity of 400,000 cpm/ml. Incubations were balanced for the inclusion of pairs of sections from each animal and performed over two to three days with the same stock solutions which were refrigerated between incubations. The hormone incubations were completed between one and eleven months following tissue collection and included controls to monitor receptor deterioration in storage.

Eleven months following tissue collection, an incubation was performed with an antibody raised in sheep against hIGF1. Five pairs of sections per animal from two of the treatment groups, low nutrition and high nutrition + BST, were used. The incubations were performed over two days, beginning the day following antibody iodination. The ^{125}I -antibody was used at 400,000 cpm/ml (160 ng/ml) $\pm$ IGF1 at 1 $\mu\text{g/ml}$.

On the basis of the images on XAR autoradiographs, periods of exposure for sections while they were under emulsion were set at 50 days for GH, 52 days for IGF1 and 58 days for the IGF1 antibody.

The incubations using different concentrations of insulin to compete for ^{125}I -IGF1 binding (p 49) were performed several months following tissue collection and the original incubation with iodinated and unlabelled IGF1. As a control for receptor deterioration during the period in frozen storage, two pairs of sections from each of five animals were also incubated with ^{125}I -IGF1 $\pm$ IGF1 as for the original incubation. Additionally, a second staining protocol was used in an attempt to further highlight the connective tissue in muscle sections. Instead of Ehrlich's haematoxylin and alcoholic eosin, Van Gieson's method was used to stain sections from the incubation with low concentrations of unlabelled insulin. This resulted in blue-black nuclei, bright red collagen and yellow counterstained cytoplasm (Lillie, 1945) but did not aid the

microscopic evaluation of sections, so staining with Ehrlich's Haematoxylin and alcoholic Eosin was reinstated for further work.

The video/computer counting system and method for random site selection used for counting autoradiographic grains in this study are described in Chapter 2 (p 52-53). The localised distribution of radiographic grains in sections from the IGF1 incubations necessitated that separate counts be made for connective tissue, muscle fibre and nerves. Six sites per section were counted for each tissue type when possible. The number of animals and sections included in the analysis for nerve binding for IGF1 were variable owing to the difficulty of locating nerves in some sections. For the GH and antibody incubations, no distinction was made between cell types for grain counting. Six sites per section were counted for GH and twelve sites per section for the antibody.

GROWTH AND CARCASS COMPOSITION

The four treatment groups were slaughtered between 09.00 and 10.00 after 42 days of treatment. Animals were transported from their pens to the research abattoir and slaughtered upon arrival. The following weights were recorded: final body weight, carcass, and liver. The left side of each carcass was minced to provide a sample for carcass composition analysis using the method of Field *et al.* (1985) as outlined in Chapter 2 (p 59).

STATISTICAL ANALYSIS

There was a large site-to-site and section-to-section variation in grain counts but the data did not require log transformation to test for treatment effects after being averaged for individual animals. The data were tested for the effects of histological quality of the section, the person doing the microscope counting, and variability between incubations on grain counts. The only significant effect on the variability of grain counts was incubation, and there was no association of this effect with aging of the incubation buffer or

incubation temperature. Variation was not reduced when the data were corrected for an effect of incubation, so analyses were performed with the unadjusted data.

Hormone concentrations were log transformed for analysis, but all other measurements were analysed without log transformation. To account for differences in body weight at the beginning of the trial, initial body weight was used as a covariate for adjustment of body weight data. Likewise, carcass weight was used as a covariate for adjustment of carcass composition data to remove the predominant effect of nutrition and possibly highlight smaller effects of BST on carcass composition. All data were analysed by ANOVA for main effects of nutrition, BST and an interaction between the two treatments.

RESULTS

PLASMA HORMONES

Plasma GH Concentrations

Plasma concentrations of GH were not affected by nutrition (Table 5.1). The increased GH in circulation with BST treatment was reflected in the results ($p \leq 0.001$), but there was no interaction between the BST and nutrition treatments on plasma GH.

Plasma IGF1 Concentrations

There was an effect of nutrition on IGF1 concentrations (Figure 5.1, $p \leq 0.001$), elevated levels being associated with good nutrition. At both levels of nutrition there was also a positive effect of BST on plasma IGF1 concentrations ($p \leq 0.01$, $p \leq 0.001$). The significant interaction between BST and nutritional treatments was reflected as a greater effect of BST on plasma IGF1 at high nutrition than at low nutrition.

Treatment	n	GH (ng/ml)
Low Nutrition	5	3.3
High Nutrition	4	3.5
Low Nutrition + BST	5	32.6
High Nutrition + BST	5	44.8
Pooled SED		2.9
Significance (Nutrition)		ns
Significance (BST)		***
Significance (Nutrition + BST)		ns

Table 5.1 Plasma concentrations of GH (ng/ml) on day 40 of treatment of animals on a low plane (1.25% of body weight/day in pellets) or a high plane (3.0% of body weight/day in pellets) of nutrition, with or without BST injection (0.25 mg/kg/day).

The values are the group means. *** $p \leq 0.001$, ns not significant (ANOVA).

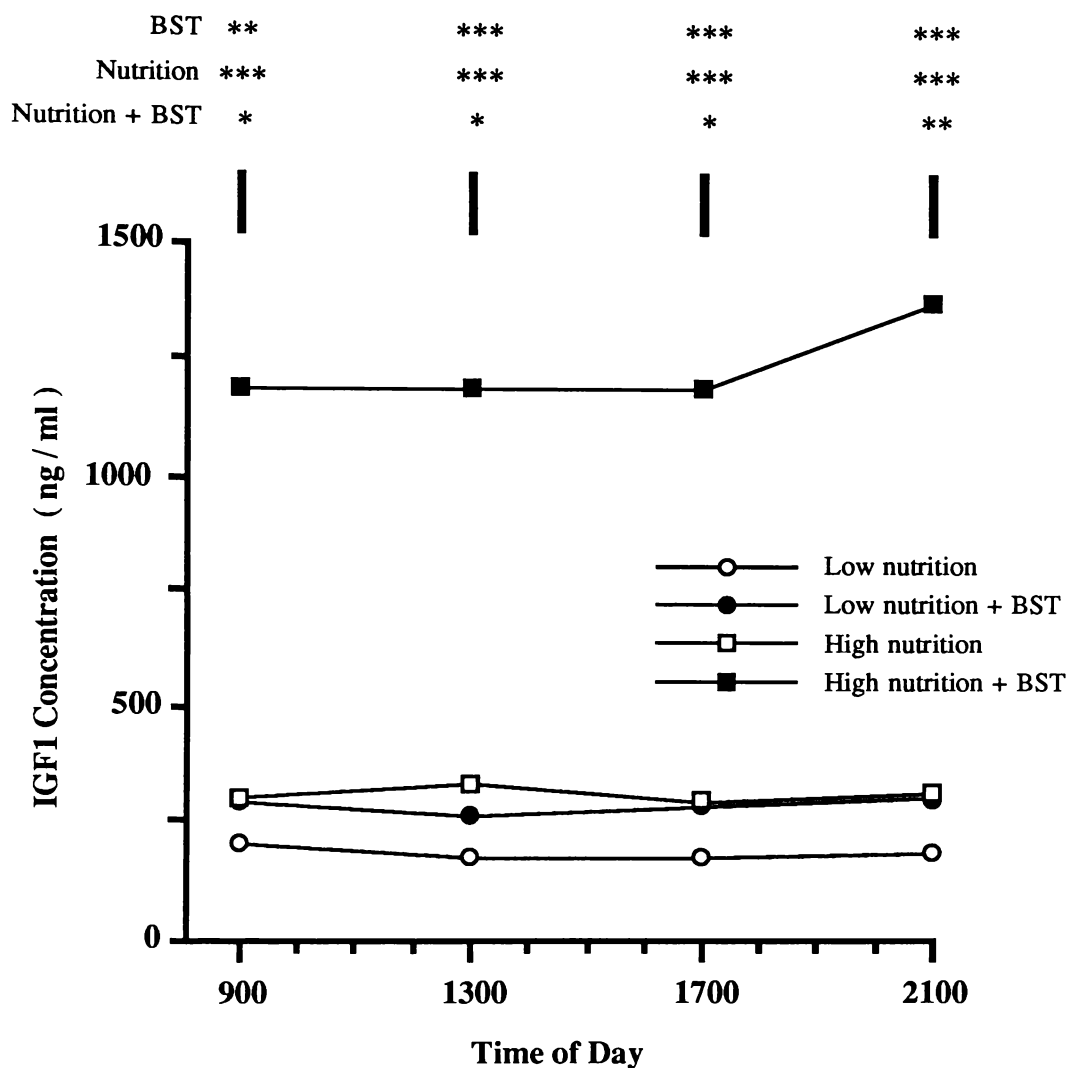


Figure 5.1 Plasma concentrations of IGF1 on day 40 of treatment of animals on low plane (1.25% of body weight/day in pellets) or a high plane (3.0% of body weight/day in pellets) of nutrition, with or without BST injection (0.25 mg/kg/day). Each data point is the group mean, with pooled SED shown above. (n=5 per group).
 * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns not significant (ANOVA).

AUTORADIOGRAPHY

The results of the autoradiographic assessment of hormone receptors are shown in Tables 5.2 to 5.5. The vertical columns of pooled SED in the Tables determine whether the difference between total and non-specific binding (specific binding) was significant within a treatment group. Horizontal rows of pooled SED test for differences between treatment groups for total, non-specific and specific binding.

GH Binding

Incubation of muscle sections with ^{125}I -GH did not demonstrate specific binding (Table 5.2).

IGF1 Binding

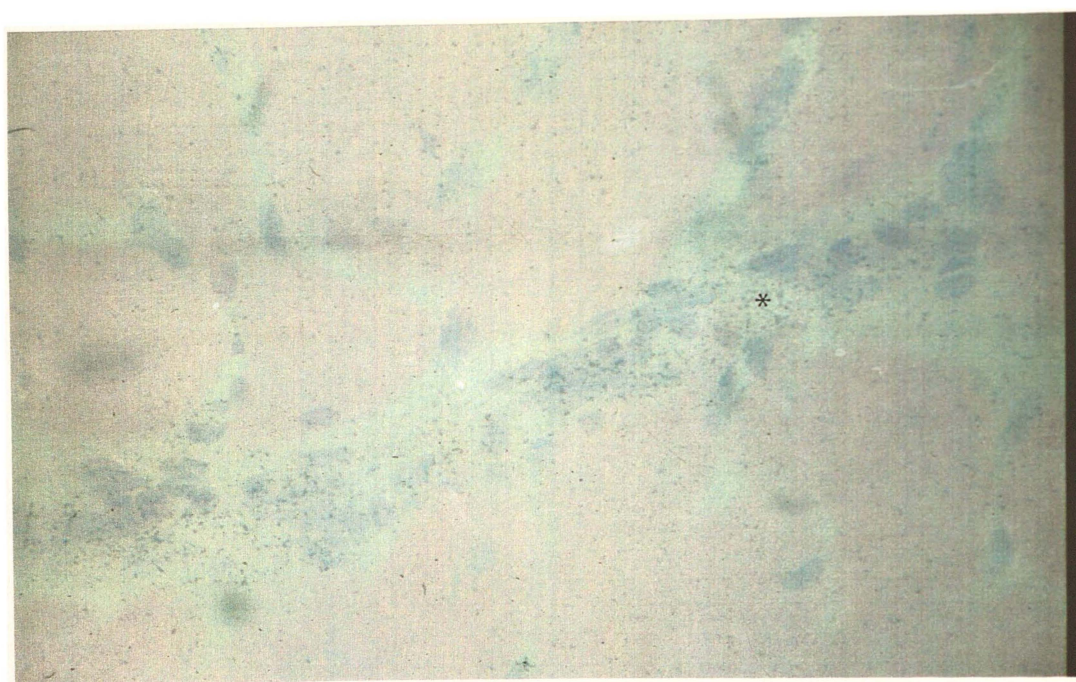
After initial viewing of the muscle sections which had been incubated with ^{125}I -IGF1, estimates of binding were made separately for connective tissue, muscle fibre and nerves (Tables 5.3, 5.4 and 5.5). This revealed localised specific binding of ^{125}I -IGF1 to the connective tissue ($p \leq 0.001$), as shown in Plate 5.1. When animals were divided into experimental groups, there was no effect of nutrition and BST treatment on the specific binding of ^{125}I -IGF1 to connective tissue (Table 5.3) and no specific binding was demonstrated in muscle fibre or nerves (Tables 5.4 and 5.5). In addition to quantitative measurements of specific binding, qualitative observations were also made. Primarily, it was noticed that blood vessels of all sizes specifically bound ^{125}I -IGF1 to a greater extent did than any other components of the muscle sections (Plates 5.2, 5.3 and 5.4). The binding appeared to be greatest in the smooth muscle band of arterioles.

To determine what type of receptor was binding ^{125}I -IGF1 in the connective tissue of muscle, two further incubations were performed using insulin as a competitor for ^{125}I -IGF1 binding. At a concentration of 10 ng/ml, insulin was expected to out-compete ^{125}I -IGF1 only for binding to the insulin receptor.

Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Low Nutrition	5	15	14	3	1.8	ns
High Nutrition	4	16	13	1	2.1	ns
Low Nutrition + BST	5	16	16	0	1.8	ns
High Nutrition + BST	5	14	16	0	1.8	ns
Pooled SED		2.5	2.7	2.8	Animals n = 19 Sections / Animal = 6 Sites / Section = 6	
Significance (Nutrition)		ns	ns	ns		
Significance (BST)		ns	ns	ns		
Significance (Nutrition + BST)		ns	ns	ns		

Table 5.2 Grain counts per unit area from muscle cryosections incubated with 125 I-GH (total binding) or 125 I-GH and unlabelled GH (non-specific binding). The cryosections were obtained from animals which had undergone 42 days of treatment on a low plane (1.25% of body weight/day in pellets) or a high plane (3.0% of body weight/day in pellets) of nutrition, with or without BST injection (0.25 mg/kg/day). The values are the group means. **ns** not significant (ANOVA).

A



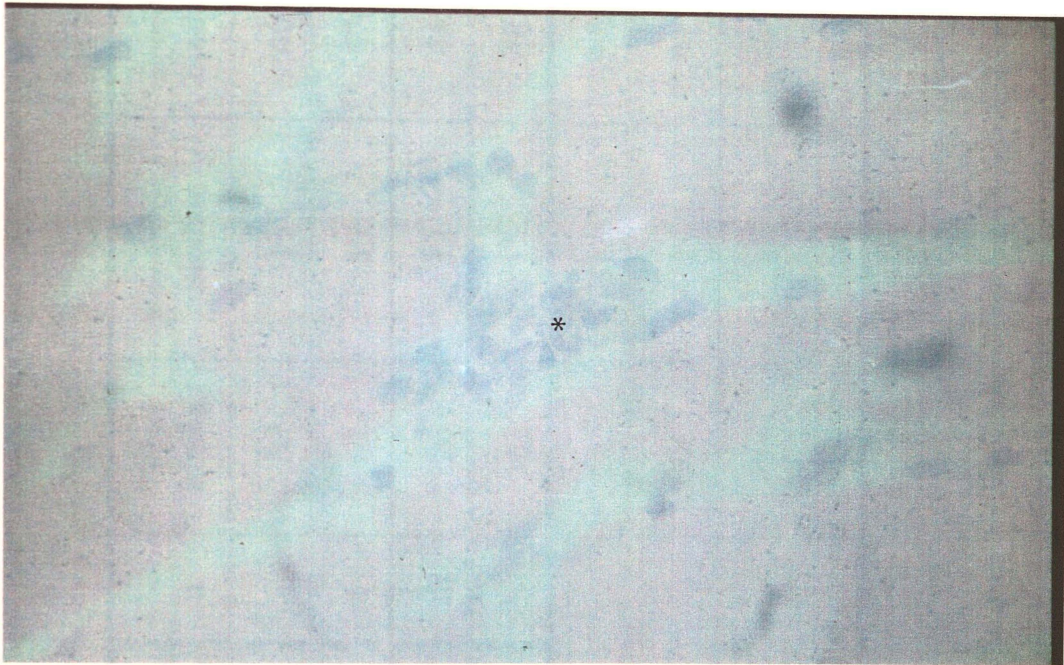
B

Plate 5.1 Total binding (A) and non-specific binding (B) of ^{125}I -IGF1 to a cryosection of skeletal muscle. Specific binding is present over connective tissue (*) but not the surrounding muscle fibre. (x400)

Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Low Nutrition	5	59	13	46	6.5	***
High Nutrition	4	67	15	52	7.3	***
Low Nutrition + BST	5	56	12	44	6.5	***
High Nutrition + BST	5	63	15	48	6.5	***
Pooled SED		10.3	1.9	9.8	Animals n = 19 Sections / Animal = 6 Sites / Section = 6	
Significance (Nutrition)		ns	*	ns		
Significance (BST)		ns	ns	ns		
Significance (Nutrition + BST)		ns	ns	ns		

Table 5.3 Grain counts per unit area from connective tissue in muscle cryosections incubated with 125 I-IGF1 (total binding) or 125 I-IGF1 and unlabelled IGF1 (non-specific binding). The cryosections were obtained from animals which had undergone 42 days of treatment on a low plane (1.25% of body weight/day in pellets) or a high plane (3.0% of body weight/day in pellets) of nutrition, with or without BST injection (0.25 mg/kg/day). The values are the group means. * $p \leq 0.05$, *** $p \leq 0.001$, ns not significant (ANOVA).

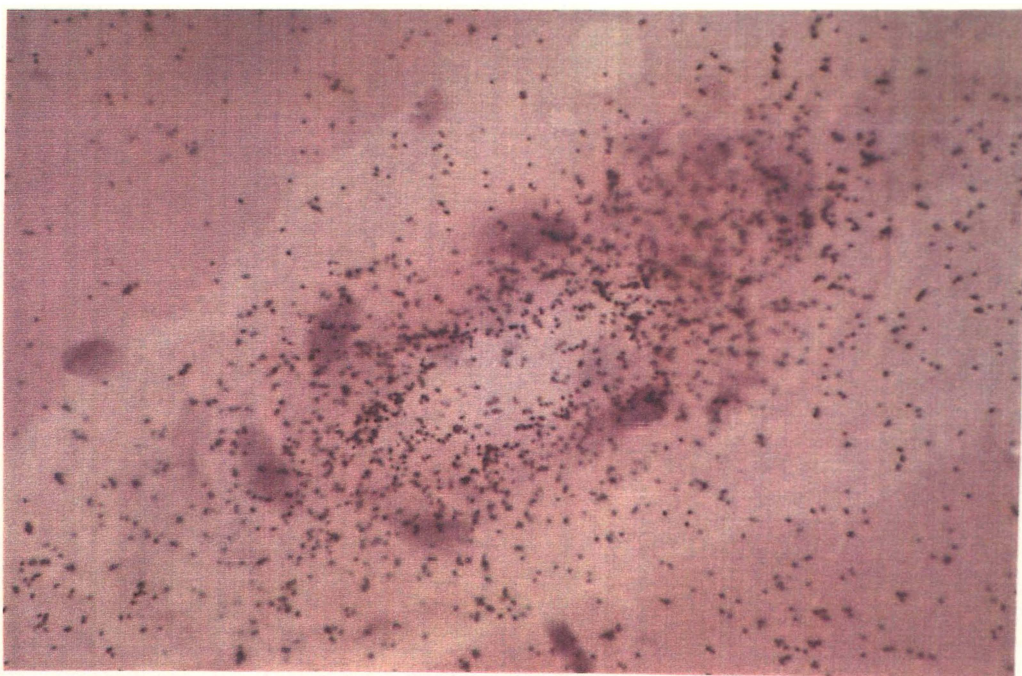
Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Low Nutrition	5	24	23	1	3.2	ns
High Nutrition	4	32	28	4	3.5	ns
Low Nutrition + BST	5	23	20	3	3.2	ns
High Nutrition + BST	5	27	25	2	3.2	ns
Pooled SED		4.0	2.9	4.7	Animals n = 19 Sections / Animal = 6 Sites / Section = 6	
Significance (Nutrition)		*	ns	ns		
Significance (BST)		ns	ns	ns		
Significance (Nutrition + BST)		ns	ns	ns		

Table 5.4 Grain counts per unit area from muscle fibre in cryosections incubated with ¹²⁵ I-IGF1 (total binding) or ¹²⁵ I-IGF1 and unlabelled IGF1 (non-specific binding). The cryosections were obtained from animals which had undergone 42 days of treatment on a low plane (1.25% of body weight/day in pellets) or a high plane (3.0% of body weight/day in pellets) of nutrition, with or without BST injection (0.25 mg/kg/day). The values are the group means. * p≤0.05, ns not significant (ANOVA).

Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Low Nutrition	5	18	21	0	3.2	ns
High Nutrition	2	17	21	0	5.0	ns
Low Nutrition + BST	3	15	16	0	4.1	ns
High Nutrition + BST	4	15	15	0	3.5	ns
Pooled SED		3.4	4.5	5.9	Animals n = 14 Sections / Animal = 4 - 6 Sites / Section = 2	
Significance (Nutrition)		ns	ns	ns		
Significance (BST)		ns	ns	ns		
Significance (Nutrition + BST)		ns	ns	ns		

Table 5.5 Grain counts per unit area from nerves in muscle cryosections incubated with 125 I-IGF1 (total binding) or 125 I-IGF1 and unlabelled IGF1 (non-specific binding). The cryosections were obtained from animals which had undergone 42 days of treatment on a low plane (1.25% of body weight/day in pellets) or a high plane (3.0% of body weight/day in pellets) of nutrition, with or without BST injection (0.25 mg/kg/day). The values are the group means. ns not significant (ANOVA).

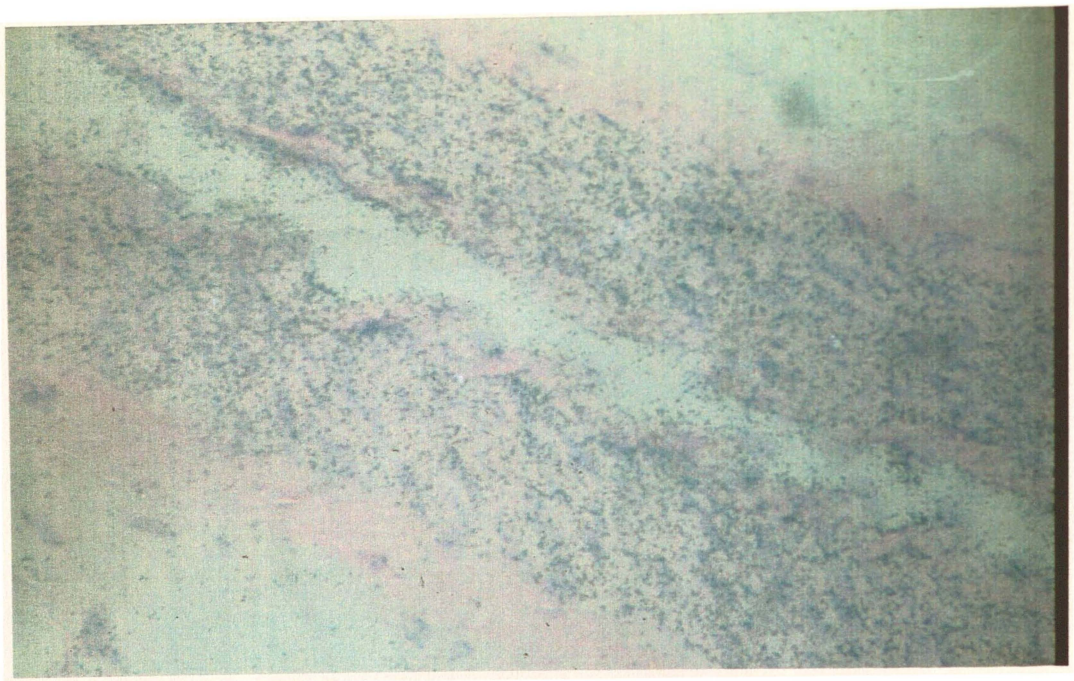
A



B

Plate 5.2 Total binding (A) and non-specific binding (B) of ^{125}I -IGF1 to a capillary within a cryosection of skeletal muscle. (x1250)

A



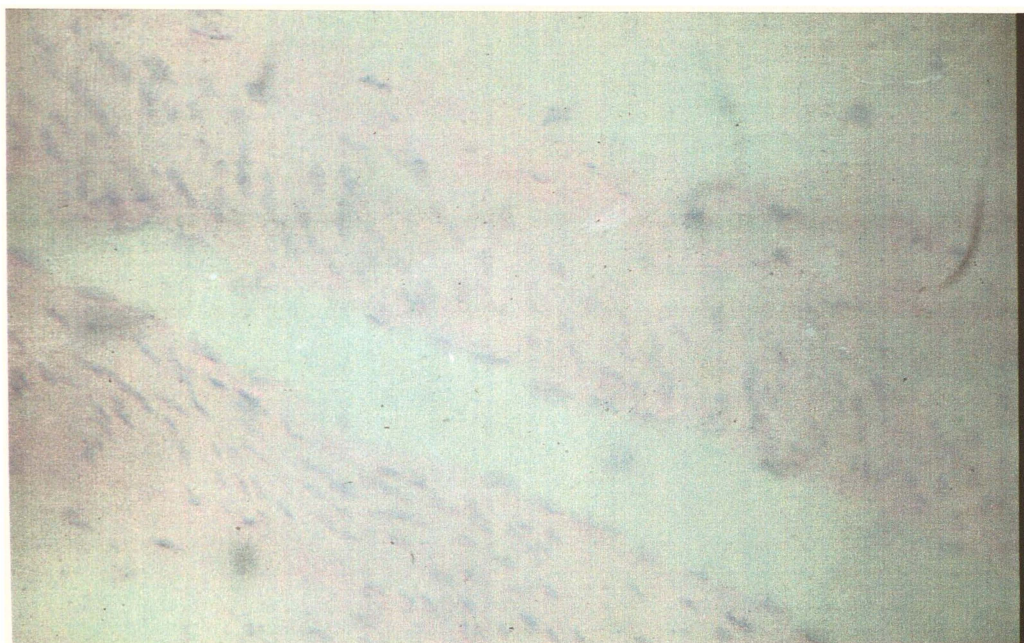
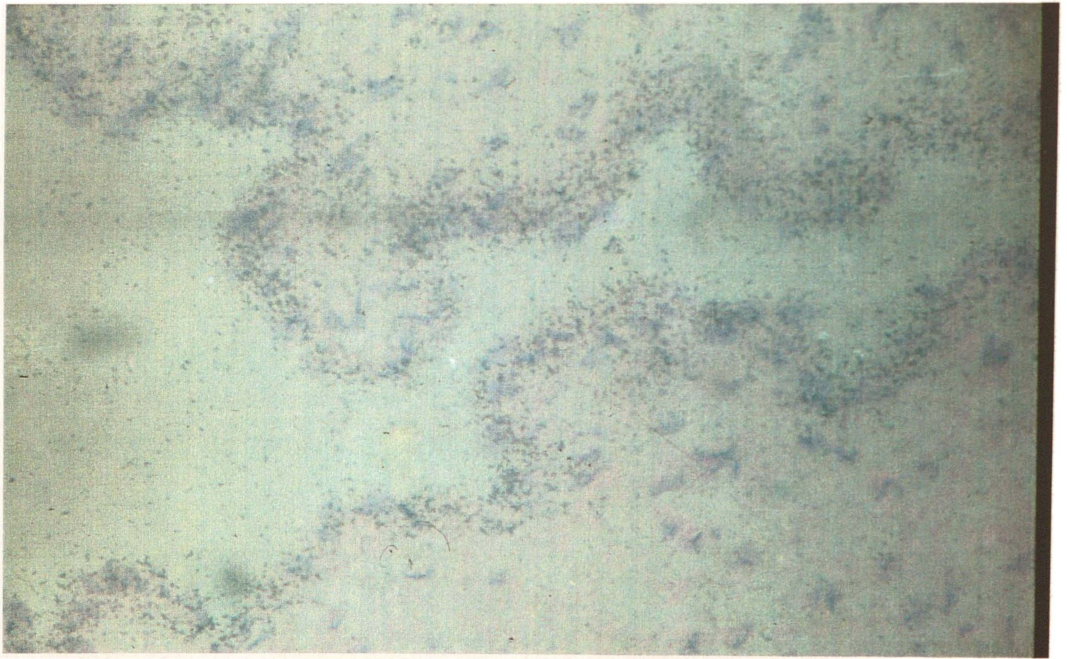
B

Plate 5.3 Total binding (A) and non-specific binding (B) of ^{125}I -IGF1 to an arteriole within a cryosection of skeletal muscle. (x400)

A



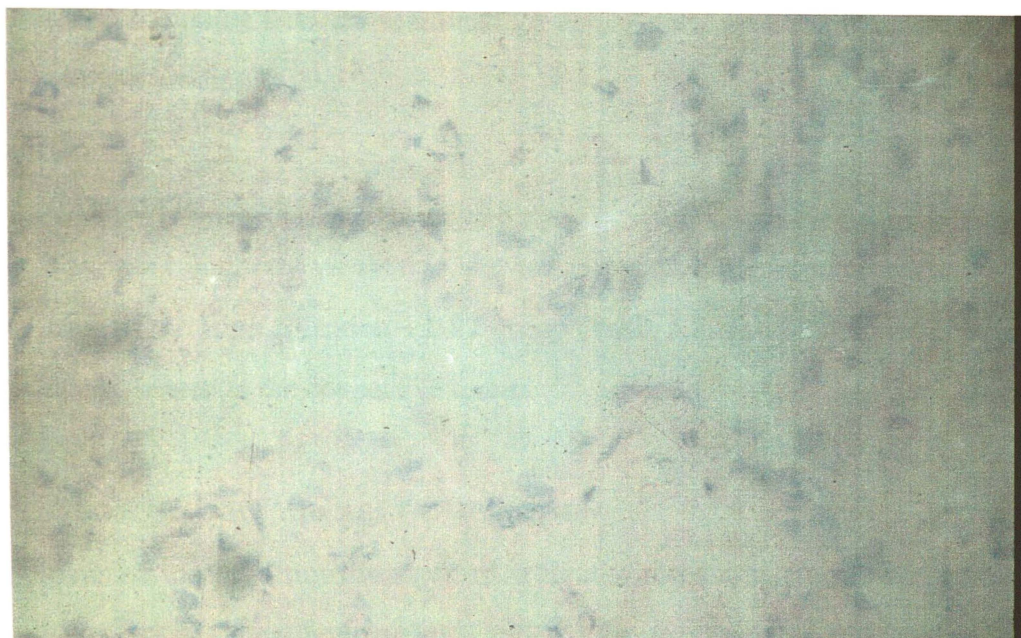
B

Plate 5.4 Total binding (A) and non-specific binding (B) of ^{125}I -IGF1 to a venule within a cryosection of skeletal muscle. (x400)

No specific binding was demonstrated in this incubation (Table 5.6), indicating that ^{125}I -IGF1 was not cross-binding significantly to insulin receptors. Data from the control incubation verify the integrity of IGF1 binding sites at the time this incubation was performed, by the demonstration of specific binding ($p \leq 0.001$).

At a concentration of 10 $\mu\text{g/ml}$, insulin competes with ^{125}I -IGF1 for binding to both the insulin and Type 1 receptor, despite having a lower affinity for the Type 1 receptor than ^{125}I -IGF1. This incubation showed effective blocking of ^{125}I -IGF1 binding by high levels of insulin (Table 5.7, $p \leq 0.01$, $p \leq 0.001$), with no significant difference in the specific binding between treatment groups. Again, the control incubation demonstrated specific binding ($p \leq 0.01$).

Immunodetection

The IGF1 antibody incubation showed marginal IGF1 immunoreactivity in the muscle fibre of the High Nutrition + BST group (Table 5.8, $p \leq 0.05$) but no specific binding was demonstrated in the connective tissue.

GROWTH AND CARCASS COMPOSITION

Animals on high nutrition gained weight while animals on low nutrition lost weight (Figure 5.2). Final body weights and carcass weights demonstrate this nutritional effect when compared with the pre-treatment slaughter group (Table 5.9, $p \leq 0.001$). There was no effect of BST treatment on the weight changes. Unlike body and carcass weights, there were effects of BST ($p \leq 0.01$) and nutrition ($p \leq 0.001$) on liver weights. BST treatment reduced the severity of weight loss in the liver with poor nutrition and increased the weight gain of the liver at high nutrition. There was no interaction between BST and nutritional treatments on liver weight.

Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Control Incubation	5	53	10	43	6.3	***
			Residual Binding	Sections / Animal = 4 Sites / Section = 6		
Low Nutrition	5	75	73	2	7.6	ns
High Nutrition	4	45	50	0	8.5	ns
Low Nutrition + BST	5	81	73	8	7.6	ns
High Nutrition + BST	5	48	42	6	7.6	ns
Pooled SED		9.9	14.2	11.4	Animals n = 19 Sections / Animal = 6 Sites / Section = 6	
Significance (Nutrition)		***	*	ns		
Significance (BST)		ns	ns	ns		
Significance (Nutrition + BST)		ns	ns	ns		

Table 5.6 Grain counts per unit area from connective tissue in muscle cryosections incubated with ¹²⁵I-IGF1 (total binding), ¹²⁵I-IGF1 and unlabelled IGF1 (non-specific binding) or ¹²⁵I-IGF1 and a physiological concentration of unlabelled insulin (residual binding). The cryosections were obtained from animals which had undergone 42 days of treatment on a low plane (1.25% of body weight/day in pellets) or a high plane (3.0% of body weight/day in pellets) of nutrition, with or without BST injection (0.25 mg/kg/day). The values are the group means. * p≤0.05, *** p≤0.001, ns not significant (ANOVA).

Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Control Incubation	5	64	10	54	11.9	**
			Residual Binding	Sections / Animal = 4 Sites / Section = 6		
Low Nutrition	5	68	32	36	8.1	***
High Nutrition	4	74	30	44	9.0	***
Low Nutrition + BST	5	65	39	26	8.1	**
High Nutrition + BST	5	65	33	32	8.1	***
Pooled SED		11.9	5.6	12.1	Animals n = 19 Sections / Animal = 6 Sites / Section = 6	
Significance (Nutrition)		ns	ns	ns		
Significance (BST)		ns	ns	ns		
Significance (Nutrition + BST)		ns	ns	ns		

Table 5.7 Grain counts per unit area from connective tissue in muscle cryosections incubated with ¹²⁵I-IGF1 (total binding), ¹²⁵I-IGF1 and unlabelled IGF1 (non-specific binding) or ¹²⁵I-IGF1 and a pharmacological concentration of unlabelled insulin (residual binding). The cryosections were obtained from animals which had undergone 42 days of treatment on a low plane (1.25% of body weight/day in pellets) or a high plane (3.0% of body weight/day in pellets) of nutrition, with or without BST injection (0.25 mg/kg/day). The values are the group means. ** p≤0.01, *** p≤0.001, ns not significant (ANOVA).

Tissue type	Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Connective Tissue	Low Nutrition	5	15	11	4	2.8	ns
	High Nutrition + BST	5	16	10	6	2.8	ns
	Pooled SED		1.6	1.8	1.9	Animals n = 10 Sections / Animal = 10 Sites / Tissue = 12	
	Significance		ns	ns	ns		

Tissue type	Treatment	n	Total Binding	Non Specific Binding	Specific Binding	Pooled SED	Significance
Muscle Fibre	Low Nutrition	5	13	9	4	1.8	ns
	High Nutrition + BST	5	14	9	5	1.8	*
	Pooled SED		1.8	1.9	1.2	Animals n = 10 Sections / Animal = 10 Sites / Tissue = 12	
	Significance		ns	ns	ns		

Table 5.8 Grain counts per unit area from connective tissue and muscle fibre in cryosections incubated with an ¹²⁵I-antibody against IGF1 (total binding) or the ¹²⁵I-antibody and unlabelled IGF1 (non-specific binding). The cryosections were obtained from animals which had undergone 42 days of treatment on a low plane of nutrition (1.25% of body weight/day in pellets) or a high plane of nutrition (3.0% of body weight/day in pellets) with BST injection (0.25 mg/kg/day). The values are the group means.

* p≤0.05, ns not significant (ANOVA).

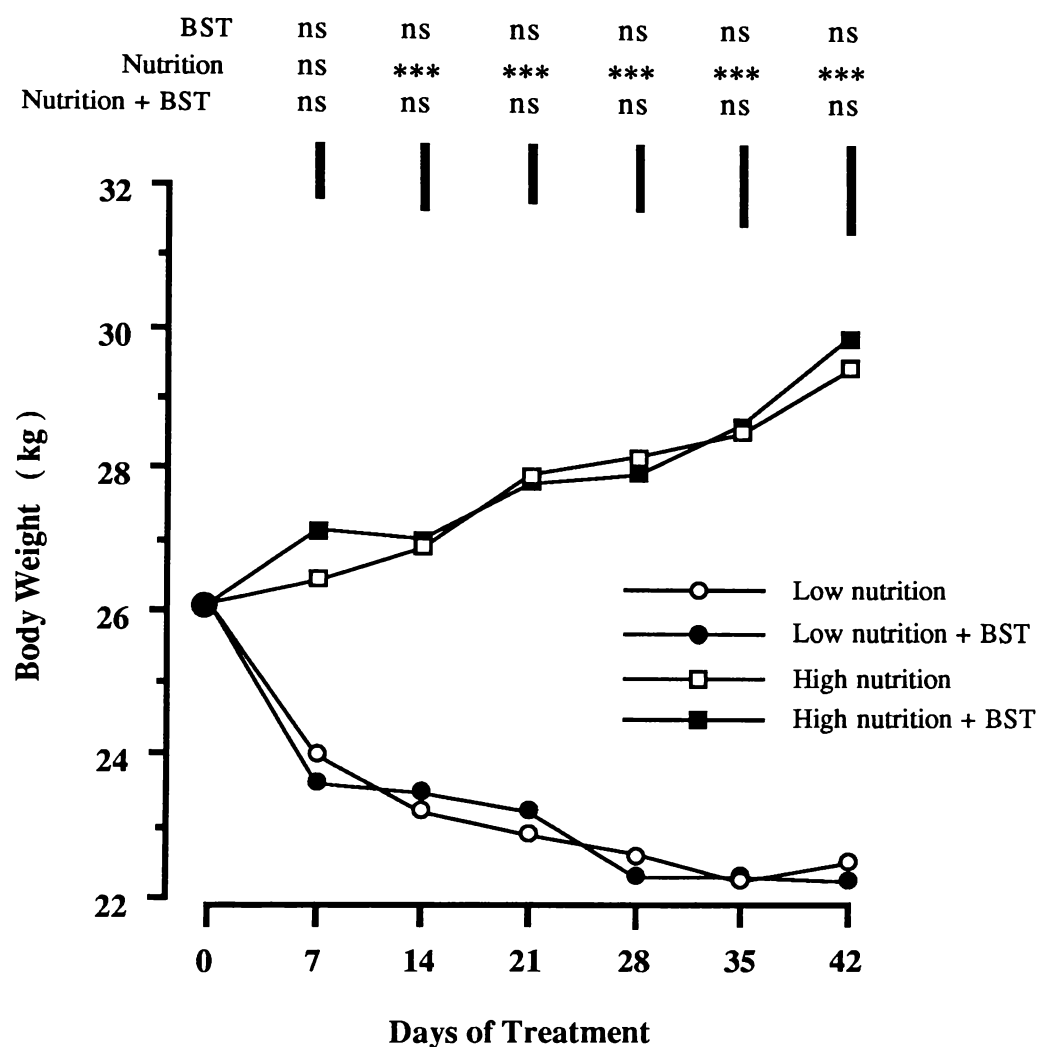


Figure 5.2 Body weight (kg) of animals throughout the period of treatment on a low plane (1.25% of body weight/day in pellets) or a high plane (3.0% of body weight/day in pellets) of nutrition, with or without BST injection (0.25 mg/kg/day). Each data point is the group mean after co-variate adjustment for differences in body weight at the beginning of the experiment. The pooled SED is shown above each data point ($n=5$ per group).

*** $p \leq 0.001$, ns not significant (ANOVA).

Treatment	n	Final Body Weight (kg)	Carcass (kg)	Liver (g)
Pre-treatment Group	5	26.7	11.9	526
Low Nutrition	5	-4.38	-1.66	-143
High Nutrition	4	2.35	0.52	42
Low Nutrition + BST	5	-3.78	-1.56	-117
High Nutrition + BST	5	3.38	1.18	111
Pooled SED		1.480	0.803	21.4
Significance (Nutrition)		***	***	***
Significance (BST)		ns	ns	**
Significance (Nutrition + BST)		ns	ns	ns

Table 5.9 Changes in weights of body components following 42 days of animal treatment on a low plane (1.25% of body weight/day in pellets) or a high plane (3.0% of body weight/day in pellets) of nutrition, with or without BST injection (0.25 mg/kg/day). The values are weight differences from the means for a group of untreated animals which was slaughtered at the beginning of the experiment.

** $p \leq 0.01$, *** $p \leq 0.001$, ns not significant (ANOVA).

Data on carcass composition (Table 5.10) show a nutritional effect of declining water ($p \leq 0.01$), fat ($p \leq 0.05$) and protein content ($p \leq 0.01$) with poor nutrition. When the data were adjusted to account for differences in carcass weight there were no differences in composition as a result of the nutritional treatment. This indicates that the carcass components were proportionally the same for animals on high or low nutrition. BST treatment increased the water content ($p \leq 0.01$) and reduced fatness ($p \leq 0.01$, $p \leq 0.001$) of carcasses.

DISCUSSION

EFFECTS OF NUTRITION AND BST ON PLASMA HORMONES

Plasma GH concentrations did not differ between animals on the high and low planes of nutrition after 40 days (Table 5.1). Patterns of GH secretion in ruminants are normally closely linked with nutrition. Under conditions of malnutrition, circulating GH concentrations are higher than under conditions of good nutrition (Hart, 1980; Weekes and Godden, 1980). Data in the current experiment suggest that the different levels of nutrition which were selected did not alter GH secretion. This contrasts with other studies in ruminants where plasma GH was sensitive to several factors regulating nutritional status. Plasma GH concentrations in cattle under diet restriction of a similar magnitude to that used in this experiment increased significantly over animals maintained on a high plane of nutrition (Breier *et al.*, 1988b). Bass *et al.*, (1987) reported increased GH concentrations in pasture-fed versus pellet-fed sheep, and Waghorn (1986) noted greater GH concentrations in sheep on a low protein pellet diet and a high protein diet with restricted intake when compared with sheep on the high protein diet at greater intake levels. It is conceivable in the current study that there was a differential response of GH secretion to the nutrition treatments earlier in the study, which had attenuated by 40 days of treatment.

Treatment	n	Water (kg)	Fat (kg)	Protein (kg)	Water [†] (kg)	Fat [†] (kg)	Protein [†] (kg)
Pre-treatment Group	5	6.95	2.26	2.11			
Low Nutrition	5	-1.28	0.32	-0.43	-0.48	0.50	-0.22
High Nutrition	4	0.10	0.52	-0.05	-0.41	0.40	-0.20
Low Nutrition + BST	5	-0.61	-0.32	-0.31	0.04	-0.18	-0.12
High Nutrition + BST	5	0.91	0.20	0.12	-0.02	-0.01	-0.14
Pooled SED		0.390	0.145	0.101	0.134	0.153	0.050
Significance (Nutrition)		**	*	**	ns	ns	ns
Significance (BST)		ns	**	ns	**	***	ns
Significance (Nutrition + BST)		ns	ns	ns	ns	ns	ns

† = Adjusted for Carcass Weight .

Table 5.10 Changes in carcass composition following 42 days of animal treatment on a low plane (1.25% of body weight/day in pellets) or a high plane (3.0% of body weight/day in pellets) of nutrition. The values are weight differences from the means for a group of untreated animals which was slaughtered at the beginning of the experiment.

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns not significant (ANOVA).

There is also the possibility of a direct effect of nutrition on plasma IGF1 concentrations and growth which by-passes a prerequisite change in GH secretion. Maiter *et al.* (1988b) found no difference in plasma GH concentrations between rats fed a low protein diet and rats on a normal protein diet, despite large differences in plasma IGF1 concentrations. Elsasser *et al.* (1989) made the same observation in steer cattle. Also, circulating GH concentrations in pregnant twin-bearing sheep were found not to differ between two planes of maternal nutrition (Blom *et al.*, 1976). These studies suggest that the extent of nutritional constraint may determine whether plasma IGF1 is depressed independently or in association with changes in plasma GH.

Plasma concentrations of IGF1 were reduced with poor nutrition (Figure 5.1) and this supports data in other studies (Bass *et al.*, 1984; Breier *et al.*, 1988b). There are a number of possible mechanisms by which this may have been achieved. Important differences in plasma GH which regulated IGF1 secretion may not have been measured due to relative insensitivity of the assay. There is also evidence that hepatic GH receptors may control IGF1 secretion. In ruminants a reduction in the extent of GH binding to hepatic membranes from poorly nourished animals has been reported (Breier *et al.*, 1988b; Bass *et al.*, 1991b) and it has also been suggested that a significant IGF1 response to good nutrition requires the presence of a specific high affinity receptor (Breier *et al.*, 1988b). A further consideration, as suggested above, is that nutrition may influence IGF1 secretion directly. Maes, Amand, Underwood *et al.* (1988), using protein-deprived hypophysectomised rats, have shown no change in the GH binding to hepatic membranes with poor nutrition, but a significantly impaired IGF1 response to GH replacement in this group. A similarity in the number of hepatic GH receptors has also been observed with rat hepatocytes from animals on two nutritional regimes, despite a decrease in plasma IGF1 with poor nutrition (Thissen *et al.*, 1990). These studies indicate that the IGF1 response to changes in nutrition may occur either as a consequence of GH regulation or independently of GH.

Both well and poorly nourished sheep in the current study had increased plasma concentrations of IGF1 when treated with exogenous GH, which is in contrast to data from bovine (Breier *et al.*, 1988b; Elsasser *et al.*, 1989) and rodent species (Maes *et al.*, 1988; Maiter *et al.*, 1988a) in which no IGF1 response to GH is elicited in malnourished animals. The size of the liver (Table 5.9) and numbers of hepatic GH receptors (Bass *et al.*, 1991b) are also increased with GH treatment at both high and low planes of nutrition. Bass *et al.* (1991a) have found that IGF1 secretion per unit weight of liver is increased with GH treatment and this is in agreement with a study demonstrating a direct relationship between GH binding and expression of hepatic IGF1 mRNA (van Neste, Husman, Möller *et al.*, 1988). Maiter *et al.* (1988a) observed no change in GH binding to hepatic membranes with GH treatment which may account for their lack of an IGF1 response to GH. The reason for these differences is possibly associated with species specificity or the time required for induction of processes involved with receptor synthesis.

The final effect which was noted was an interaction between BST and nutrition whereby the stimulation of IGF1 was significantly greater in well nourished than in poorly nourished animals. This corroborates the suggestion of either impaired IGF1 secretion or reduced numbers of GH receptors in malnutrition, but overall the data from the current experiment indicate that treatment with exogenous GH has the capacity to override the depressive effects of poor nutrition and enhance the effects of good nutrition on the GH-IGF axis.

EFFECTS OF NUTRITION AND BST ON RECEPTOR POPULATIONS IN SKELETAL MUSCLE

GH receptors were not identified in skeletal muscle in the current study, although stimulation of the uptake of amino acids by GH in cultured myotubes has been reported (Ewton and Florini, 1980) and receptors have been localised in skeletal muscle immunohistochemically (Lobie *et al.*, 1990c). No distinction was made for counting

radiographic grains associated with different cell types as there was no obvious localisation of GH binding to particular tissues within the section, such as was seen with IGF1 binding. Small differences in binding between connective tissue and muscle fibre may not have been measured as connective tissue makes up only a small proportion of the muscle section. The random selection of sites for counting probably over-sampled muscle fibre and possibly failed to measure binding in connective tissue. Additionally, estimates of binding using radiolabelled hormone to detect receptors is relatively less sensitive than use of a specific antibody against the receptor (A. Nilsson, *personal communication*).

A further consideration is that ovine *M. semi-membranosus* might be a muscle which is relatively insensitive to GH. In cattle the fibre composition of the muscle is mixed, with approximately equal proportions of Type 1 and Type 2 fibres (Hunt and Hedrick, 1977). In rats, hypersecretion of GH results in selective hypertrophy of Type 1 fibres (Pryor-Jones and Jenkins, 1980) and the proportion of Type 1 fibres in muscles of mixed composition is regulated by GH (Ayling *et al.*, 1989). Selection of another ovine muscle may have demonstrated the presence of specific binding of GH.

No available binding sites for IGF1 in muscle fibre were observed (Table 5.4) despite earlier reports that IGF1 binds to and has metabolic effects on differentiating muscle cells (Dodson, Allen, Shimizu *et al.*, 1987; Beguinot *et al.*, 1988). Muscle cell hyperplasia is thought to be completed before birth in meat animals, with further muscle growth being achieved through hypertrophy of fibres (Dayton and Hathaway, 1989). Possibly the presence of Type 1 receptors in muscle is restricted to an earlier stage of development than that of the animals used in the current experiment.

The binding of ^{125}I -IGF1 to Type 1 receptors in connective tissue was not changed by GH treatment and nutritional constraint of the animals (Table 5.3). With IGF binding systems *in vitro*, down-regulation of Type 1 receptors by prior exposure to IGF1 and GH

has been reported (de Vroede *et al.*, 1984; Watanabe *et al.*, 1985). This phenomenon is not a consequence of receptor occupancy by the unlabelled hormone (Rosenfeld and Dollar, 1982; Freemark, Comer, Mularoni *et al.*, 1990). The result of IGF1 binding in the current experiment suggests either that plasma concentrations of GH and IGF1 were not being transported from the general circulation to the connective tissue, or that the connective tissue was insensitive to the experimental manipulations - possibly an age-related decline in tissue sensitivity as suggested earlier for muscle fibre. The latter suggestion is more likely. A direct effect of GH on IGF1 mRNA in skeletal muscle has been demonstrated (Isgaard *et al.*, 1989) and when IGF1 is administered to sheep it is cleared to peripheral tissues and lymph (Hodgkinson, Bass, Davis *et al.*, 1990), suggesting that the availability of either hormone to the connective tissue would not be limited. Independently of regulatory effects of GH and IGF1 on Type 1 receptors, any significant occupation of receptors by plasma IGF1 would also have resulted in altered binding of IGF1 *in vitro*, probably in a manner reflecting the widely divergent plasma concentrations of IGF1 for the treatment groups if the receptor population was stable. Therefore it is possible that there are relatively few Type 1 receptors in the skeletal muscle of older lambs. Changes in muscle which were associated with the experimental treatment and which also altered IGF1 status may instead be mediated through IGF1 cross-binding to the insulin receptor.

Significant IGF immunoreactivity has been reported in histological sections in peripheral nerves (Anderson *et al.*, 1986; Jennische *et al.*, 1987) and foetal skeletal muscle (Hill *et al.*, 1989). These observations, in contrast to the lack of IGF1 binding sites found for muscle fibre and nerves in the present study (Table 5.5), potentially allow two conclusions to be drawn: either there are no receptors which will bind IGF1 in the equivalent sheep tissues, or the receptors are pre-occupied with endogenous hormone and therefore remain undetected. Incubation of sections with an IGF1 antibody failed to indicate which explanation is more likely. The results equivocally suggest IGF1 immunoreactivity in muscle fibre but total binding counts were extremely low and,

qualitatively, no immunoreactivity was obvious in blood vessels. High levels of IGF immunoreactivity in blood vessels have previously been documented for rats (Hansson *et al.*, 1987) and cattle (Glimm *et al.*, 1988). Further characterisation of the IGF1 antibody subsequently revealed that the ability to detect natural IGF1 was extremely limited. A recombinant IGF1 was used as the immunogen and this antibody was generated against the N-Met region of the recombinant hormone (Hodgkinson *et al.*, 1989b), a component which is not present in natural IGF1.

The highest densities of grain counts in sections were observed in blood vessels. Specific receptors with high affinity for IGF1 have been described previously in the smooth muscle (Bornfeldt, Arnqvist, Dahlkvist *et al.*, 1988) and endothelial cells (Bar, Boes and Sandra, 1985; Bar, Siddle, Dolash *et al.*, 1988) of blood vessels. IGF1, IGF2 and insulin have metabolic effects in blood vessel cell systems, stimulating protein synthesis, amino acid transport and glucose uptake (Pfeifle, Ditschuneit and Ditschuneit, 1982; Bar *et al.*, 1988). Binding of IGFs to smooth muscle cells in culture also appear similar to that in non-cultured cells (Bornfeldt *et al.*, 1988), with insulin showing poor potency for blocking IGF1 binding (Pfeifle *et al.*, 1982; Bornfeldt *et al.*, 1988). The same result was observed in the present experiment with little reduction in grain density following co-incubation of ^{125}I -IGF1 with a pharmacological concentration of insulin. In addition to suggesting a significant concentration of Type 2 receptors and/or IGF binding proteins, both of which are found in endothelial cells (Bar, Boes and Yorek, 1986; Bar, Booth, Boes *et al.*, 1989), it is also possible that the Type 1 receptor in blood vessels has a greater affinity for IGF1 than does the receptor in connective tissue. This could be related to the postulated function of blood vessels as storage and processing sites for plasma IGFs destined for the extracellular space in target tissues (Bar *et al.*, 1986).

EFFECTS OF NUTRITION AND BST ON GROWTH AND CARCASS COMPOSITION

There are several reports of BST treatment affecting growth in ruminants (Johnsson *et al.*, 1985; Bass *et al.*, 1991a), following early studies where equivocal effects were noted with administration of an extracted hormone preparations. Muir *et al.* (1983) postulated several reasons for the inconsistent results: purity of the GH preparation, use of heterologous GH, administration method, dosage, species for treatment, animal age and variability in response. Some of these problems have been resolved with the development of bacterially derived recombinant products.

A metabolic consequence of GH action is to direct nutrient availability away from adipose tissue and to make it available to other target tissues (Etherton *et al.*, 1987). This is supported by results from work *in vitro* with ovine adipose tissue (Vernon, 1982) which suggest that GH acts as an insulin antagonist, retarding insulin-stimulated fatty acid synthesis and stimulating lipolysis. The reduction of fat in carcasses from sheep treated with GH is well documented (Muir *et al.*, 1983; Butler-Hogg and Johnsson, 1987). In the current experiment this was the predominant effect of GH treatment on the carcass. Independently of the effects of nutrition on carcass composition, GH treatment also increased water content in the carcass as reported by others (Pell and Bates, 1987; Bass *et al.*, 1991a).

CONCLUSION

The treatment of lambs with prolonged nutritional restriction and exogenous GH resulted in an alteration of growth. The measurements of plasma GH and IGF1 suggest that some effects may have been mediated by the GH-IGF axis, but there was no strong evidence that this was the predominant hormonal system controlling the animals metabolic responses to the experimental treatment.

Although IGF1 concentrations in plasma were manipulated to a large extent by both nutrition and BST, this apparently had little consequence on growth as indicated by the small changes in carcass composition and the limited presence of Type 1 receptors in skeletal muscle.

The anatomical resolution provided by receptor analysis has been important in allowing the localisation of Type 1 receptors to connective tissue, and the demonstration of stability of this population. These data support the idea that skeletal muscle from the lambs of this age, in general or in *M. semi-membranosus* in particular, was insensitive to stimulation by IGF1. A further useful observation was that the binding of IGF1 to blood vessels was characteristically different from that in connective tissue. Observations such as this begin to reveal how much more complicated hormone interactions with target cells are than simple binding to a homologous or heterologous receptor and initiation of a post-receptor event.

In conclusion, the manipulation of growth in this experiment was probably not mediated through the GH-IGF axis. The age of the animals may not have been optimal and the marked difference in carcass fat was probably a direct consequence of BST stimulation of adipose tissue.

CHAPTER SIX:

THE EFFECTS OF FASTING ON GH AND IGF CONCENTRATIONS IN PLASMA, GH AND IGF RECEPTORS IN SKELETAL MUSCLE, AND GROWTH

INTRODUCTION

The impact of restricted nutrition on the physiology of growth is a topic of considerable clinical and agricultural investigation. Growth cannot occur in the absence of adequate nutrition, and the effect of fasting on the endocrinology of growth has been investigated in a number of species. GH concentrations are usually elevated during fasting (Merimee and Fineberg, 1974; Harvey, Scanes, Chadwick *et al.*, 1978; Ho *et al.*, 1988). In sheep, the reports have been contradictory. Some have concluded that there is no change in plasma GH with fasting (Wallace and Bassett, 1970; McAtee and Trenkle, 1971; Trenkle, 1976; Van Maanen, McCutcheon and Purchas, 1989; Freemark *et al.*, 1990) while others have observed elevated concentrations (Bassett *et al.*, 1971; Driver and Forbes, 1981). These reports have been based on widely differing sampling regimes, ranging from single sample estimates to prolonged serial sampling at intervals of several minutes.

In fasted rats, plasma IGF1 levels decline (Davenport *et al.*, 1990) in association with reduced numbers of GH receptors in the liver (Postel-Vinay *et al.*, 1982; Thissen *et al.*, 1990). Expression of mRNA in liver for the GH receptor (Straus and Takemoto, 1990) and IGF1 protein (Lowe *et al.*, 1989; Davenport *et al.*, 1990; Straus and Takemoto, 1990) is also reduced. Plasma IGF1 concentrations also appear to be reduced in fasted

sheep (Hodgkinson *et al.*, 1987; Freemark *et al.*, 1990). The response of plasma IGF2 to fasting has not been conclusively established. It has been reported that plasma IGF2 is depressed in fasted pigs (Buonomo *et al.*, 1988), but foetal and adult rats do not show a change of plasma IGF2 or IGF2 mRNA (Davenport *et al.*, 1990).

Lowe *et al.* (1989) have investigated the response of Type 1 receptors and the content of Type 1 receptor and IGF1 mRNA in central and peripheral tissues from fasted rats. They have demonstrated that the tissues respond differentially and do not necessarily show a coordinated change in receptors, receptor mRNA and expression of IGF1 mRNA. The regulation of ovine receptors for the growth hormones under conditions of fasting has not been reported, but insulin binding to muscle membranes increases following nutrient restriction (Grizard, 1983).

The aim of the present experiment was to document the response of plasma GH, IGF1 and IGF2 to prolonged fasting, and to evaluate whether the experimental treatment regulated receptor availability for these hormones in skeletal muscle.

MATERIALS AND METHODS

MATERIALS

The pellets were purchased from NRM Feeds Limited and composed of lucerne (60%), barley (30%), linseed oil (5%) and molasses (5%).

ANIMALS AND TREATMENT

Ten Suffolk x Coopworth ewe lambs of six months old were penned individually indoors and fed sheep pellets *ad libitum*. Water was available *ad libitum* and the pellets were given at 09.00 daily. During a pre-treatment period of three weeks the animals were weighed once weekly and daily feed intakes were recorded. At the end of this time they

were randomly allocated to one of two treatment groups which were balanced for liveweight. The treatments were as follows:

Fasting (n=5):

Feeding (n=5): continued *ad libitum* feeding of pellets

Treatment was for five days, during which time the concentrations of glucose and non-esterified fatty acids in plasma were monitored to assess physiological stress, particularly of the fasting animals. On the sixth morning the animals were transported to the research abattoir for slaughter. Carcass and liver weights were recorded and pieces of *M. biceps femoris* dissected and frozen in liquid nitrogen for autoradiography.

BLOOD SAMPLING

Two days before treatment began, the animals were fitted with an indwelling catheter into one jugular vein. The catheters were flushed each day with heparinised saline (25 000 IU/l) to keep them patent. The day before treatment a series of 10 ml blood samples was collected into heparinised tubes for GH assay. The samples were taken at fifteen minute intervals from 08.30 to 14.00, then hourly until 22.00. Between samples the catheters were flushed with saline containing 15 000 IU heparin/l. A 10 ml sample was also collected at 08.30 into tubes containing EDTA for IGF assay. This sampling regime was repeated on the first and final days of treatment. Between the intensive bleeds (days two to four) the catheters were flushed daily and the 08.30 sample collected for IGF assay. Samples were kept on ice and centrifuged under refrigeration as soon as possible. The plasma samples were then frozen at -20°C until assay.

PLASMA HORMONE ASSAYS

GH (Spencer *et al.*, 1991, p 56)

Samples were assayed for GH within four months of collection. The assay consisted of incubating plasma or standards with an oGH antiserum raised in rabbits and then the addition of ¹²⁵I-GH. There was a further incubation with rabbit antiserum raised in goats,

followed by PEG precipitation, centrifugation and aspiration of the supernatant and gamma counting of the pellets.

IGF1 (Hodgkinson *et al.*, 1991, p 57)

Samples were assayed for IGF1 within one month of collection. The assay followed acid/ethanol extraction of plasma to remove the hormone from endogenous plasma binding proteins. Extracted plasmas or standards were initially incubated with oIGF1 antiserum raised in rabbits and then with ^{125}I -IGF1. A mixture of rabbit antiserum raised in goats, normal rabbit serum and PEG was added for a further period of incubation, PEG then used for precipitation and the tubes centrifuged, the supernatant aspirated and the pellets gamma counted.

IGF2 (Hodgkinson *et al.*, unpublished method, p 58)

Samples for IGF2 were assayed within four months of collection. The plasmas were extracted to remove plasma binding proteins as with IGF1 samples. The assay consisted of the simultaneous addition to standards or extracted plasmas of ^{125}I -IGF2 and a mouse monoclonal antiserum against IGF2. Following the initial incubation, mouse antiserum raised in sheep and PEG were added and the samples precipitated after a further incubation. Finally the tubes were centrifuged, the supernatant aspirated and the pellets gamma counted.

AUTORADIOGRAPHY

Muscle samples from two animals in each treatment group were used for autoradiography incubations. The animals with IGF1 concentrations closest to their respective group means on the final day of treatment were selected for this work. To eliminate the variation between incubations and the necessity for pairing sections, the six sections for any one radiolabelled hormone incubation were cut and thaw-mounted onto a gelatinised slide in two groups of three - one group for total binding and the other for non-specific or residual binding. Eleven such slides were produced for each animal and

were incubated within one week of being cut. The reduced number of slides enabled all treatments with a radiolabelled hormone to be processed in a single incubation. This in turn allowed the pooling of grain counts from a larger number of sections which were used to determine total binding.

It was noted in some earlier autoradiographs (Chapter 5) that freezing damage to the section was substantial. Typically, this was seen as holes in the tissue structure. One step in section preparation which may have contributed to this was the immediate replacement of thaw-mounted sections into the freezing cabinet of the cryostat. If moisture from the thawing of sections was still present as they refroze, the formation of ice crystals could disrupt the tissue morphology. In this experiment, the mounted sections were dried as others have done (Lesniak *et al.*, 1988; Smith *et al.*, 1988) for five to ten minutes before being replaced in the freezer to assess whether this improved histological quality.

Incubations were performed with three radiolabelled hormones and various unlabelled hormones. ^{125}I -GH (2.5 ng/ml, 400,000 cpm/ml) was incubated with sections alone or in combination with GH (1 $\mu\text{g/ml}$) or Prl (1 $\mu\text{g/ml}$). GH cross-binds, with lower affinity, to the Prl receptor in ovine liver (Harding, Webb and Wallis, 1986). If specific binding of GH is demonstrated then the incubation with unlabelled Prl will determine whether the binding is to the GH or Prl receptor. ^{125}I -IGF1 (1.4 ng/ml, 400,000 cpm/ml) was incubated with sections alone and with IGF1 (1 $\mu\text{g/ml}$), IGF2 (1 $\mu\text{g/ml}$) or insulin (10 ng/ml or 10 $\mu\text{g/ml}$). Likewise ^{125}I -IGF2 (1.4 ng/ml, 400,000 cpm/ml) was incubated with or without IGF2 (1 $\mu\text{g/ml}$), IGF1 (1 $\mu\text{g/ml}$) or insulin (10 ng/ml or 10 $\mu\text{g/ml}$).

The IGF1 antibody used for immunodetection was raised in sheep against N-Met hIGF1. Unlike the antibody used earlier (p 163), this antiserum recognised the natural IGF1 peptide as well as the recombinant hormone (S Hodgkinson, *personal communication*). The antibody was incubated with sections at an activity level of 400,000 cpm/ml

(160 ng/ml) either alone or having been blocked by pre-incubation with N-Met IGF1 (1 µg/ml). The IGF2 antiserum for immunodetection was a monoclonal antibody raised in mice against hIGF2 (Amano Pharmaceutical Company). It was also incubated with sections at an activity level of 400,000 cpm/ml (160 ng/ml), either alone or having been blocked by pre-incubation with IGF2 (1 µg/ml).

The incubation protocols for hormone (p 48) and antibody (p 49) autoradiographs were followed as previously described. Briefly, sections were pre-incubated with buffer, then incubated with the radiolabelled hormone or antibody and unlabelled hormone when appropriate. Excess solution was washed from the sections with buffer before they were fixed. The incubation with antibody proceeded similarly with the exception that sections were fixed before incubation. Autoradiographs were produced using XAR film and NTB emulsion (p 51-52). The periods of emulsion exposure were 28 days for ¹²⁵I-GH and ¹²⁵I-IGF1 autoradiographs, seven days for ¹²⁵I-IGF2 autoradiographs and 42 days for the ¹²⁵I-antibody autoradiographs. Grain counts were taken from six sites per tissue type per section.

STATISTICAL ANALYSIS

Plasma concentrations of GH, IGF1 and IGF2 were analysed for treatment differences during the experiment without log transformation. The data from autoradiography were also analysed without log transformation, with the exception of a combined analysis which was done for muscle fibre and connective tissue for the incubation with the ¹²⁵I-antibody. Initial bodyweight was used as a covariate for adjustment and analysis of the data recorded at slaughter. All data were analysed by ANOVA.

RESULTS

PLASMA HORMONES

Plasma GH Concentrations

Within 24 hours of having food withdrawn, GH levels in the fasted group increased (Table 6.1). This was not significant in comparison with the fed group but was a significant increase compared with pre-treatment concentrations of GH in the fasted group ($p \leq 0.05$, pooled SED = 0.61 ng/ml). By the final day of treatment, GH concentrations in the fasted group had remained elevated over the pre-treatment levels ($p \leq 0.01$, pooled SED = 0.58 ng/ml) and were also greater than GH concentrations in the fed group ($p \leq 0.01$). On the final day of treatment GH levels of the fed group were also lower than pre-treatment levels ($p \leq 0.05$, pooled SED = 0.58 ng/ml).

Plasma IGF1 Concentrations

IGF1 concentrations were similar between the treatment groups until the fourth day of fasting (Figure 6.1). At this time, levels in the fasted group were less than in the fed group and remained this way for the duration of the experiment. IGF1 concentrations were not significantly less in the fasted than the fed group until the final day of treatment ($p \leq 0.05$), despite this being apparent two days earlier. Variation in IGF1 data from the fasted group ranged from 60% less than to 453% greater than variation in the fed group. This would be directly reflected in the limited statistical significance of the differences which were obtained.

Plasma IGF2 Concentrations

There was no significant difference in IGF2 concentrations between fasted and fed animals throughout the experiment (Figure 6.2). Data were highly variable within groups, both between animals and between daily samples from an animal.

		GH (ng / ml)		
Treatment	n	Pre-treatment	Day 1	Day 6
Fasted	5	4.2	6.0*	7.1**
Fed	5	4.5	4.3	2.6*
Pooled SED		0.60	1.26	0.99
Significance		ns	ns	**

Table 6.1 Plasma concentrations of GH (ng/ml) on the day before, the first and final days of animal treatment on a regime of fasting or feeding *ad libitum*. The values are group means. * $p \leq 0.05$, ** $p \leq 0.01$, ns not significant (ANOVA).

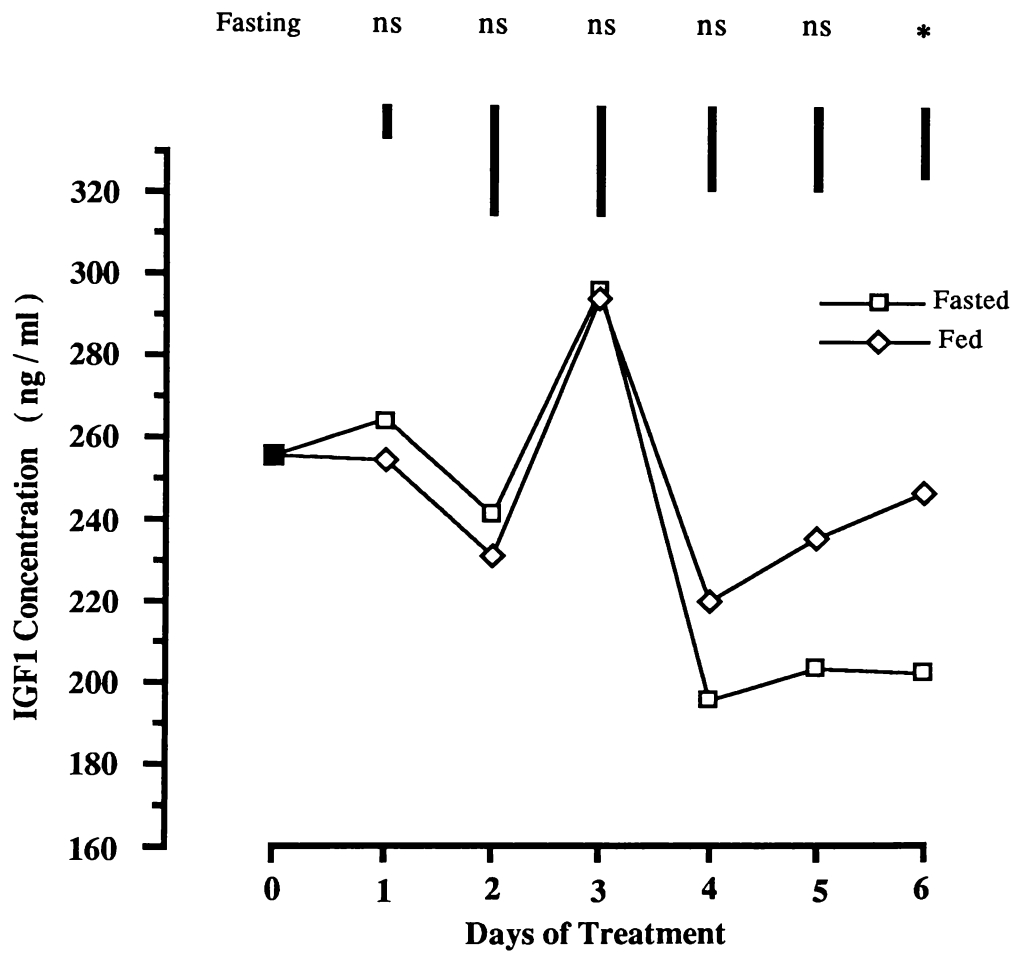


Figure 6.1 Plasma concentrations of IGF1 (ng/ml) following fasting or feeding *ad libitum* of animals for five days. Each data point is the group mean, with the pooled SED shown above (n=5 per group).

* $p \leq 0.05$, ns not significant (ANOVA).

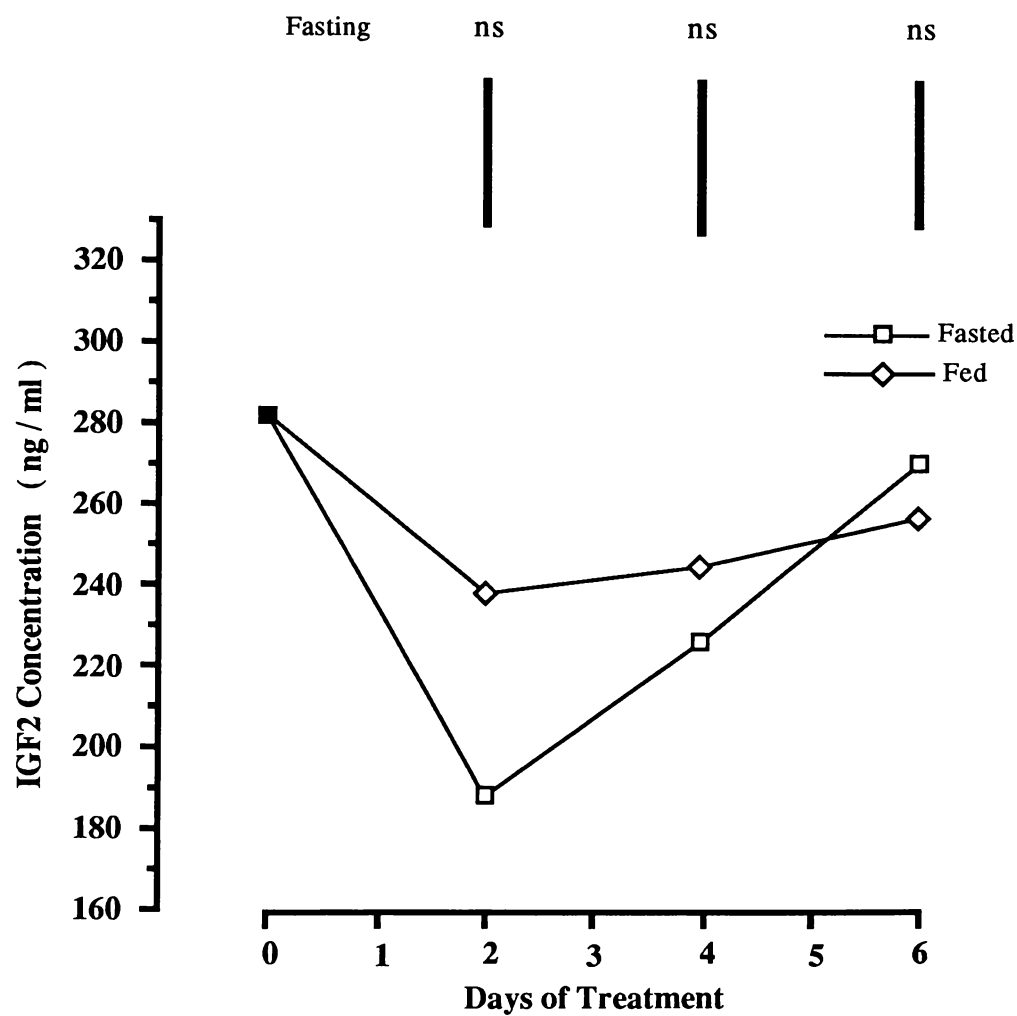


Figure 6.2 Plasma concentrations of IGF2 (ng/ml) following fasting or feeding *ad libitum* of animals for five days. Each data point is the group mean with the pooled SED shown above (n=5 per group). ns not significant (ANOVA).

AUTORADIOGRAPHY

As in Chapter 5, data are presented separately for grain counts over regions of muscle fibre and connective tissue. There was no improvement in the histological quality of autoradiographs with drying of the cryosections following cutting.

GH Binding

No specific binding of ^{125}I -GH to muscle fibre (Table 6.2) or connective tissue (Table 6.3) was demonstrated. A comparison of the counts for each tissue type shows non-specific binding of GH to be relatively high and much greater with muscle fibre than connective tissue.

IGF1 Binding

Specific binding of ^{125}I -IGF1 to muscle fibre was present (Table 6.4, $p \leq 0.05$) but there was no significant difference between the fed and fasting animals with respect to the amount of specific binding. IGF2 was as potent as IGF1 in competing for ^{125}I -IGF1 binding (Table 6.5, $p \leq 0.05$). High concentrations of insulin also competed effectively for ^{125}I -IGF1 binding sites (Table 6.6, $p \leq 0.05$) but low concentrations of insulin did not (Table 6.7), suggesting that the binding was to the Type 1 IGF receptor.

Specific binding of ^{125}I -IGF1 to connective tissue was also demonstrated (Table 6.8, $p \leq 0.05$, $p \leq 0.01$) and differed between the treatments ($p \leq 0.05$). The fasted group bound more than twice as much labelled IGF1 as the fed group. Unlabelled IGF2 was not quite as effective as a competitor for ^{125}I -IGF1 binding in connective tissue (Table 6.9) as it was in muscle fibre. Specific binding with competition by IGF2 was only demonstrated for the fasted group ($p \leq 0.05$). The high concentration of insulin (Table 6.10) was equipotent with IGF2, showing specific binding of ^{125}I -IGF1 in the fasted group only ($p \leq 0.05$). Competition for specific binding sites by low levels of insulin was negligible (Table 6.11), again suggesting that the binding was to the Type 1 IGF receptor.

Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Fasted	2	89	116	0	14.9	ns
Fed	2	104	98	6	14.9	ns
Pooled SED		2.0	20.6	21.1	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		*	ns	ns		

Table 6.2 Grain counts per unit area from muscle fibre in cryosections incubated with 125 I-GH (total binding) or 125 I-GH and unlabelled GH (non-specific binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means . * $p \leq 0.05$, ns not significant (ANOVA).

Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Fasted	2	47	53	0	8.3	ns
Fed	2	48	44	4	8.3	ns
Pooled SED		4.1	15.1	11.8	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		ns	ns	ns		

Table 6.3 Grain counts per unit area from connective tissue in muscle cryosections incubated with ^{125}I -GH (total binding) or ^{125}I -GH and unlabelled GH (non-specific binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means. ns not significant (ANOVA).

Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Fasted	2	51	20	31	4.6	*
Fed	2	43	19	24	4.6	*
Pooled SED		7.2	1.9	6.6	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		ns	ns	ns		

Table 6.4 Grain counts per unit area from muscle fibre in cryosections incubated with ¹²⁵I-IGF1 (total binding) or ¹²⁵I-IGF1 and unlabelled IGF1 (non-specific binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means. * p≤0.05, ns not significant (ANOVA).

Treatment	n	Total Binding	Residual Binding	Specific Binding	Pooled SED	Significance
Fasted	2	51	22	29	4.8	*
Fed	2	43	20	23	4.8	*
Pooled SED		7.2	1.6	6.6	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		ns	ns	ns		

Table 6.5 Grain counts per unit area from muscle fibre in cryosections incubated with ¹²⁵I-IGF1 (total binding) or ¹²⁵I-IGF1 and unlabelled IGF2 (residual binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means. * p≤0.05, ns not significant (ANOVA).

Treatment	n	Total Binding	Residual Binding	Specific Binding	Pooled SED	Significance
Fasted	2	51	14	37	5.3	*
Fed	2	43	10	33	5.3	*
Pooled SED		7.2	0.3	7.5	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		ns	**	ns		

Table 6.6 Grain counts per unit area from muscle fibre in cryosections incubated with 125 I-IGF1 (total binding) or 125 I-IGF1 and a pharmacological concentration of unlabelled insulin (residual binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means. * $p \leq 0.05$, ** $p \leq 0.01$, ns not significant (ANOVA).

Treatment	n	Total Binding	Residual Binding	Specific Binding	Pooled SED	Significance
Fasted	2	51	42	9	7.9	ns
Fed	2	43	40	3	7.9	ns
Pooled SED		7.2	8.3	11.1	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		ns	ns	ns		

Table 6.7 Grain counts per unit area from muscle fibre in cryosections incubated with 125 I-IGF1 (total binding) or 125 I-IGF1 and a physiological concentration of unlabelled insulin (residual binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means.
 ns not significant (ANOVA).

Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Fasted	2	41	10	31	2.5	**
Fed	2	20	7	13	2.5	*
Pooled SED		3.8	1.8	3.6	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		*	ns	*		

Table 6.8 Grain counts per unit area from connective tissue in muscle cryosections incubated with ¹²⁵I-IGF1 (total binding) or ¹²⁵I-IGF1 and unlabelled IGF1 (non-specific binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means. * p≤0.05, ** p≤0.01, ns not significant (ANOVA).

Treatment	n	Total Binding	Residual Binding	Specific Binding	Pooled SED	Significance
Fasted	2	41	13	28	3.3	*
Fed	2	20	7	13	3.3	ns
Pooled SED		3.8	0.9	4.6	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		*	*	ns		

Table 6.9 Grain counts per unit area from connective tissue in muscle cryosections incubated with 125 I-IGF1 (total binding) or 125 I-IGF1 and unlabelled IGF2 (residual binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means. * $p \leq 0.05$, ns not significant (ANOVA).

Treatment	n	Total Binding	Residual Binding	Specific Binding	Pooled SED	Significance
Fasted	2	41	16	25	3.3	*
Fed	2	20	6	14	3.3	ns
Pooled SED		3.8	0.8	4.6	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		*	**	ns		

Table 6.10 Grain counts per unit area from connective tissue in muscle cryosections incubated with 125 I-IGF1 (total binding) or 125 I-IGF1 and a pharmacological concentration of unlabelled insulin (residual binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means. * $p \leq 0.05$, ** $p \leq 0.01$, ns not significant (ANOVA).

Treatment	n	Total Binding	Residual Binding	Specific Binding	Pooled SED	Significance
Fasted	2	41	24	17	10.5	ns
Fed	2	20	19	1	10.5	ns
Pooled SED		3.8	11.2	14.8	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		*	ns	ns		

Table 6.11 Grain counts per unit area from connective tissue in muscle cryosections incubated with 125 I-IGF1 (total binding) or 125 I-IGF1 and a physiological concentration of unlabelled insulin (residual binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means.

* $p \leq 0.05$, ns not significant (ANOVA).

There were no large blood vessels in sections to enable the confirmation of high levels of specific binding of ^{125}I -IGF1 as noted in the previous experiment (p 142). The most striking difference between these data for ^{125}I -IGF1 binding and that presented in Chapter 5 was the presence of binding to muscle fibre. To test whether a maturational change was responsible, grains were counted in muscle autoradiographs from new-born ewe lambs following incubation with radiolabelled IGF1 and unlabelled IGF1 (1 $\mu\text{g/ml}$). These data (Table 6.12) show the specific binding of ^{125}I -IGF1 to muscle fibre ($p \leq 0.05$) and connective tissue ($p \leq 0.001$). The specific binding was significantly greater in connective tissue than fibre ($p \leq 0.01$).

IGF2 Binding

^{125}I -IGF2 did not bind specifically to muscle fibre (Table 6.13), but did bind to connective tissue (Table 6.14, $p \leq 0.01$). There was no significant difference in the binding between fed and fasted groups although binding in the fasted group was lower. Unlabelled IGF1 (Table 6.15), high concentrations of insulin (Table 6.16) and low concentrations of insulin (Table 6.17) were all ineffective competitors for the specific binding of ^{125}I -IGF2 to connective tissue, suggesting that the binding was to the Type 2 IGF receptor. As with ^{125}I -IGF1, greatest binding in sections for ^{125}I -IGF2 was to blood vessels. The binding was not displaceable by unlabelled hormones other than IGF2. In some sections there was a greater grain density over veins than nearby arterioles but this observation was not consistent. Binding of ^{125}I -IGF2 also differed visually from binding of ^{125}I -IGF1 in some regions of connective tissue, where it was localised to a greater extent over the nuclei.

Immunodetection

The immunodetection of endogenous IGF1 was marginal. When counts were analysed separately, specific binding of antibody in muscle fibre (Table 6.18) and connective tissue (Table 6.19) approached significance for the fed animals. This was substantiated when the counts were analysed without distinguishing the tissue types (Table 6.20, $p \leq 0.01$).

Tissue type	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Fibre	2	53	35	18	5.8	*
Connective tissue	2	68	17	51	5.8	***
Pooled SED		3.8	3.8	8.2	Animals n = 2 Sections / Animal = 6 Sites / Section = 6	
Significance		**	**	**		

Table 6.12 Grain counts per unit area from muscle fibre and connective tissue in muscle cryosections incubated with 125 I-IGF1 (total binding) or 125 I-IGF1 and unlabelled IGF1 (non-specific binding). The cryosections were obtained from new-born lambs. The values are group means. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (ANOVA).

Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Fasted	2	45	48	0	7.5	ns
Fed	2	43	40	3	7.5	ns
Pooled SED		6.9	11.4	10.6	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		ns	ns	ns		

Table 6.13 Grain counts per unit area from muscle fibre in cryosections incubated with ¹²⁵I-IGF2 (total binding) or ¹²⁵I-IGF2 and unlabelled IGF2 (non-specific binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means. **ns** not significant (ANOVA).

Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Fasted	2	78	16	62	6.2	**
Fed	2	98	12	86	6.2	**
Pooled SED		8.3	4.9	8.8	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		ns	ns	ns		

Table 6.14 Grain counts per unit area from connective tissue in muscle cryosections incubated with ¹²⁵I-IGF2 (total binding) or ¹²⁵I-IGF2 and unlabelled IGF2 (non-specific binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means. ** p≤0.01, ns not significant (ANOVA).

Treatment	n	Total Binding	Residual Binding	Specific Binding	Pooled SED	Significance
Fasted	2	78	82	0	12.1	ns
Fed	2	98	119	0	12.1	ns
Pooled SED		8.3	22.0	17.1	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		ns	ns	ns		

Table 6.15 Grain counts per unit area from connective tissue in muscle cryosections incubated with 125 I-IGF2 (total binding) or 125 I-IGF2 and unlabelled IGF1 (residual binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means. ns not significant (ANOVA).

Treatment	n	Total Binding	Residual Binding	Specific Binding	Pooled SED	Significance
Fasted	2	78	93	0	10.6	ns
Fed	2	98	89	9	10.6	ns
Pooled SED		8.3	21.0	15.0	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		ns	ns	ns		

Table 6.16 Grain counts per unit area from connective tissue in muscle cryosections incubated with 125 I-IGF2 (total binding) or 125 I-IGF2 and a pharmacological concentration of unlabelled insulin (residual binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means. ns not significant (ANOVA).

Treatment	n	Total Binding	Residual Binding	Specific Binding	Pooled SED	Significance
Fasted	2	78	76	2	17.3	ns
Fed	2	98	112	0	17.3	ns
Pooled SED		8.3	32.0	24.5	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		ns	ns	ns		

Table 6.17 Grain counts per unit area from connective tissue in muscle cryosections incubated with 125 I-IGF2 (total binding) or 125 I-IGF2 and a physiological concentration of unlabelled insulin (residual binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means. ns not significant (ANOVA).

Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Fasted	2	84	71	13	8.9	ns
Fed	2	115	77	38	8.9	ns
Pooled SED		6.7	5.9	12.5	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		*	ns	ns		

Table 6.18 Grain counts per unit area from muscle fibre in cryosections incubated with an ¹²⁵I-antibody against IGF1 (total binding) or the ¹²⁵I-antibody and unlabelled IGF1 (non-specific binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means.

* p≤0.05, ns not significant (ANOVA).

Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Fasted	2	89	67	22	9.5	ns
Fed	2	103	68	35	9.5	ns
Pooled SED		4.3	9.8	13.4	Animals n = 4 Sections / Animal = 6 Sites / Section = 6	
Significance		ns	ns	ns		

Table 6.19 Grain counts per unit area from connective tissue in muscle cryosections incubated with an 125 I-antibody against IGF1 (total binding) or the 125 I-antibody and unlabelled IGF1 (non-specific binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means.
 ns not significant (ANOVA).

Treatment	n	Total Binding	Non-specific Binding	Specific Binding	Pooled SED	Significance
Fasted	2	86	69	20	10.2	ns
Fed	2	109	72	37	10.2	**
Pooled SED		7.4	7.4	14.4	Animals n = 4 Sections / Animal = 6 Sites / Section = 12	
Significance		ns	ns	ns		

Table 6.20 Grain counts per unit area from a combination of muscle fibre and connective tissue in muscle cryosections incubated with an 125 I-antibody against IGF1 (total binding) or the 125 I-antibody and unlabelled IGF1 (non-specific binding). The cryosections were obtained from animals which were fasted or fed *ad libitum* for five days. The values are group means. ** $p \leq 0.01$, ns not significant (ANOVA).

The combined analysis failed to demonstrate immunodetection of IGF1 in the fasted group. There was also no localisation of antibody binding to blood vessels.

Autoradiography with the IGF2 antibody was unsuccessful. On sections for total and non-specific binding, grains in the emulsion were clumped rather than evenly distributed and this was also apparent in the XAR autoradiographs. There was no pattern to the clumping, which occurred over the sections and also directly on the microscope slide. Clumping was not reduced by filtration of buffers, increasing the concentration of mouse IgG in the blocking buffer, or fixation of sections after incubation. Therefore, these autoradiographs were excluded from analysis.

DISSECTION

By the final sample on the fifth day of treatment, glucose levels were less in fasted than fed animals (Fasted, 57.7 ± 2.39 , Fed, 81.8 ± 2.82 , $X \pm \text{SEM}$ mg/100 ml) but non-esterified fatty acids were greater (Fasted, 0.67 ± 0.21 , Fed, 0.14 ± 0.03 , $X \pm \text{SEM}$ mEq/l) indicating the continued mobilisation of fat from adipose depots. Packed cell volumes from blood samples collected immediately before slaughter were comparable between treatments (Fasted, 23 ± 1.8 , Fed, 26 ± 1.1 , $X \pm \text{SEM}$ %) with no suggestion of the fasted animals having become dehydrated. A treatment effect on carcass weight was apparent after initial liveweight was used as a covariate for adjustment (Table 6.21) with fasted animals having lighter carcasses than fed animals ($p \leq 0.01$). An effect on liver weight was also present with livers from fasted animals being approximately 35% lighter than livers from fed animals ($p \leq 0.001$). These data verify the severe impact of fasting on growth.

Treatment	n	Carcass	Carcass[†]	Liver	Liver[†]
Fasted	5	10.84	10.67	0.48	0.48
Fed	5	12.23	12.40	0.74	0.74
Pooled SED		1.137	0.370	0.030	0.030
Significance		ns	**	***	***

† = Adjusted for carcass weight

Table 6.21 Weights of body components from animals which were fasted or fed *ad libitum* for five days. The values are group means. ** p<0.01, *** p<0.001, ns not significant (ANOVA).

DISCUSSION

EFFECTS OF FASTING ON PLASMA HORMONES

The most immediate effect of fasting was an elevation in plasma GH concentrations within fourteen hours of withdrawing pellets. This became more pronounced by the final day of treatment (Table 6.1). Some of the contradictions concerning GH levels during fasting may be resolved by investigating the sampling frequency and duration of fasting in various studies. Studies in which single or limited and infrequent samples were taken showed effects of fasting on GH (Bassett *et al.*, 1971; Schaefer, Krishnamurti, Heindze *et al.*, 1984) and also showed no effect (Trenkle, 1976; Van Maanen *et al.*, 1989; Freemark *et al.*, 1990). Driver and Forbes (1981) used a more frequent sampling regime, similar to the current experiment, and demonstrated episodic release of GH with peaks preceding and troughs in conjunction with ingestion. The depressed GH concentrations associated with feeding have been suggested to occur as a result of reduced pituitary sensitivity to stimulation by GRH (Trenkle, 1989). Most animals showed no synchrony of release, but with fasting an increase in peak and trough levels was evident within a few hours (Driver and Forbes, 1981). This corroborates data presented from the current experiment where an increase in GH was detected on the first day of treatment. Driver and Forbes suggested that mean plasma concentrations may not be a valid measurement of GH, but on the basis of data in the current experiment, means are acceptable in situations where sampling frequency is high and the experimental treatment is prolonged.

The Driver and Forbes study imposed a ten hour fast, while fasting in this experiment continued for 120 hours. The other studies have looked at periods of 48 or 72 hours fasting. On the basis of all the studies, there was no evidence of a differential effect of short term or long term fasting on GH concentrations. Elevated GH levels were not attenuated by the fifth day of treatment, the increase from pre-treatment concentrations being more significant than on day one. By this stage, the difference between treatment

groups was also significant. On the final day of treatment, GH levels had declined in the fed sheep from pre-treatment concentrations. This may have been a consequence of the intensive handling of animals, but behavioural stress has previously been shown to have no effect on plasma GH in sheep (Cronin, Siegal, Moberg *et al.*, 1981). The average refusal of pellets on the day before treatment began was 31% of the pellets offered, compared with 24% on the final day of treatment. The relatively higher consumption of pellets later in the experiment may be closely associated with levels of GH secretion, a concept explored by Driver and Forbes (1981) who noted immediate declines in GH at the onset of even short periods of feeding. Other factors which cannot be discounted are the sensitivity of radioimmunoassay and the sex, age and breed of sheep.

The physiological consequence of continuously elevated GH concentrations may be associated with homeostasis. Exogenous administration of GH to sheep results in a reduction of carcass fat (p 157), so GH secreted during fasting may act similarly as an anti-lipogenic agent for mobilising stored fatty acids. Another documented effect of GH on ovine adipose tissue, antagonism of insulin-stimulated lipogenesis (Vernon, 1982) may also have been in effect, the final result also being low levels of lipogenesis. However, it was assumed that plasma insulin in the fasted animals was low at the conclusion of the experiment. This was suggested by the reduced plasma concentrations of glucose in the fasted group.

Plasma concentrations of IGF1 responded more slowly to fasting than did concentrations of GH. IGF1 concentrations were significantly lower by the last day of treatment (Figure 6.1) although the divergence between treatment groups had begun earlier. Others working with fasted sheep have reported declining IGF1 concentrations by two days (Hodgkinson *et al.*, 1987) and three days (Freemark *et al.*, 1990) of treatment. Unlike the report of an increased half life of plasma GH with fasting (Trenkle, 1976), there was no change in the half life of plasma IGF1 (Hodgkinson *et al.*, 1987). This may be associated with the formation of stable IGF1-plasma binding protein complexes in

plasma, despite variations in the absolute levels of assayable IGF1. There was no evidence of a treatment effect after two days in the current experiment but fasted IGF1 levels appeared lower after three days. Both other studies (Hodgkinson *et al.*, 1987; Freemark *et al.*, 1990) reported reductions in the range of 35 to 45% compared with 20% in the current experiment. Freemark *et al.* (1990) worked with pregnant ewes while Hodgkinson *et al.* worked with wethers approximately three months older than those in this experiment.

Using the Hodgkinson study for comparison, a sex difference in IGF1 sensitivity between pre-pubertal females and castrated males is unlikely. There may be a maturational increase in sensitivity for both the onset and extent of IGF1 reduction with fasting, although the ontogenetic development of IGF1 in plasma from sheep is characterised by a decline from six weeks old (Gluckman and Butler, 1983). In a similar study with fasted rats, Thissen *et al.* (1990) suggested less depression in IGF1 with advancing age, and the initiation of reduced IGF1 secretion was slower in mice which were almost fully grown than in younger mice (McKnight and Goddard, 1989).

While increased GH is clearly a stimulus for increased plasma IGF1 levels in sheep (p 139) there is an uncoupling of this relationship with fasting. The increase in GH with fasting is followed at least two days later by a decline in plasma IGF1, suggesting perhaps an over-riding control of IGF1 secretion by nutritional status. GH receptors may be important in the nutritional regulation of IGF1 secretion. GH binding to liver membrane preparations from sheep, while being regulated by plasma GH, is influenced to a greater extent by nutritional status of the animal (Bass *et al.*, 1991b). Under severe nutritional constraint such as fasting, the two endocrine responses may be independent and quite dissociated from their growth-promoting actions and interactions in well nourished animals. It has already been suggested that elevated GH may increase nutrient availability to the animal. Likewise, reduced IGF1 may act to depress non-essential metabolic and growth processes during nutritional deficit (Merimee, Zapf and Froesch,

1982). Merimee *et al.*, 1982 have suggested that the substrates present during fasting *e.g.* free fatty acids and ketones, may down-regulate IGF1 secretion. It has also been postulated that reduced IGF1 in rodents may be permissive in allowing tissue catabolism during fasting (O'Sullivan *et al.*, 1989) and that the action of somatomedin inhibitors may contribute to altered tissue processes (Phillips, Goldstein and Gavin, 1988).

Plasma measurements of IGF2 indicated no change between or within groups throughout fasting (Figure 6.2). This is in agreement with results obtained in adult and foetal rats after a 24 hour fast (Davenport *et al.*, 1990) and in humans following five days of fasting (Davenport, Svoboda, Koerber *et al.*, 1988). The data contrast with the finding of reduced IGF2 concentrations following fasting for 48 hours in pigs (Buonomo *et al.*, 1988) and 24 hours in new-born rats (Phillips, Drakenberg, Persson *et al.*, 1989). Buonomo *et al.* (1988) have reported a developmental difference in the IGF2 response to GH treatment whereby there was no stimulation in younger pigs but significant stimulation in older animals, and the data provided by Davenport *et al.* (1988) and Phillips *et al.* (1989) may also demonstrate an effect of ontogenetic development of a plasma IGF2 response to fasting. It may be that the period of greatest sensitivity to regulation by fasting, independently or possibly as a consequence of GH stimulation, is developmentally later than IGF1 sensitivity or endogenously elevated IGF2 concentrations. Plasma IGF2 concentrations are high in the sheep foetus and decline to adult levels at birth (Gluckman and Butler, 1983), but this does not preclude the possibility of later development of further physiological regulation of IGF2 in plasma.

As expected, five days of fasting resulted in reduced carcass and liver weights (Table 6.1). Reduced bodyweight after a 24 hour fast has been reported in mice (O'Sullivan *et al.*, 1990) and reduced body and liver weights in rats (Thissen *et al.*, 1990). To some extent the reduced size of liver may account for the decline in plasma IGF1 if this was the primary source of hormone, but the same principle does not apply for the plasma IGF2 concentrations. If a comparison is made of the depression in IGF1 (20%)

and reduction in liver weight (35%) then it may be speculated either that IGF1 secretion per unit weight of liver increased, or that other peripheral sites of synthesis which contribute to plasma concentrations were less sensitive to fasting.

EFFECTS OF FASTING ON RECEPTOR POPULATIONS IN SKELETAL MUSCLE

No specific binding of ^{125}I -GH was demonstrated in muscle fibre (Table 6.3), connective tissue (Table 6.4) or to blood vessels. Conclusions which may be drawn from the ^{125}I -GH incubations are limited primarily because of the relatively large amounts of non-specific binding. The conclusion of an absence of GH receptors available for binding is more likely to be safely made when binding of the radiolabelled hormone is relatively low. This has been demonstrated with autoradiographs of antler which also show no specific binding of GH. Total and non-specific binding sections of deer antler have a mean grain count per unit area of approximately eleven after two weeks exposure under emulsion (J. Elliott and J. Oldham, unpublished data). This compares with counts of approximately 48 for connective tissue and 100 for muscle fibre after a one week exposure period in the current experiment. Furthermore, the lack of GH receptors demonstrated autoradiographically in the antler has been substantiated in binding experiments with membrane preparations (Ambler, Breier, Klempt *et al.*, 1990). The problem of sensitivity of detection appears to originate from two sources. Firstly the tissue type, with muscle fibre binding hormones non-specifically to a greater extent than either connective tissue or blood vessels. Additionally, characteristics of the ^{125}I -GH also result in greater non-specific binding than ^{125}I -IGF1 or ^{125}I -IGF2 to the same tissue components of sections.

GH receptors have recently been demonstrated immunohistochemically in a number of peripheral tissue systems; growth plate chondrocytes (Werther *et al.*, 1990), male and female reproductive tracts (Lobie *et al.*, 1990a), and cultured chondrocytes (Nilsson, Edén and Isaksson, 1989). Supporting the immunodetection of GH receptors on growth plate chondrocytes was the autoradiographic demonstration of specific ^{125}I -GH binding

to cultured chondrocytes (Werther *et al.*, 1990). Non-specific binding comprised 48% of total binding, and the authors concluded that immunodetection of GH receptors was the technique with greater sensitivity. Non-specific binding also comprised 40% of total ^{125}I -GH binding in cultured fibroblasts (Murphy *et al.*, 1983).

There are reports of the peripheral distribution of GH receptors (Lobie *et al.*, 1990a), direct actions of GH on skeletal muscle (Isgaard *et al.*, 1989), and regulation of hepatic GH receptors and GH receptor mRNA (Straus and Takemoto, 1990; Thissen *et al.*, 1990) by fasting, but no demonstration yet of ^{125}I -GH binding in skeletal muscle. Specific binding of ^{125}I -GH to cultured fibroblasts has been reported (Murphy *et al.*, 1983), suggesting that connective tissue might be a potential site for localisation of ^{125}I -GH binding in muscle sections. GH receptor immunoreactivity is also intense in the endothelial component of blood vessels (Lobie *et al.*, 1990b). The failure to demonstrate ^{125}I -GH binding autoradiographically in the current experiment is likely to have been a consequence of technical considerations associated with the tissue and use of radiolabelled hormone, and a low abundance of receptors in skeletal muscle (Lobie *et al.*, 1990a).

Specific binding of ^{125}I -IGF1 was demonstrated in muscle fibre (Table 6.5) and connective tissue (Table 6.9). The results of various incubations with unlabelled hormones (IGF2, Table 6.6 and Table 6.10; high concentrations of insulin, Table 6.7 and Table 6.11; low concentrations of insulin, Table 6.8 and Table 6.12), together suggest that the binding is to a Type 1 receptor. This is evaluated by the extent of cross-binding of hormones to heterologous receptors and is used as a diagnostic feature of receptor identification in conventional biochemical binding assays (p 49).

The demonstration of ^{125}I -IGF1 binding to muscle fibre contrasts with results obtained in earlier incubations (p 142) which conclusively showed no binding, but supports the report of metabolic effects of IGF1 on ovine myoblasts in culture (Harper *et al.*, 1987).

Further incubations with muscle sections from much younger lambs verified the presence of specific binding sites for ^{125}I -IGF1 (Table 6.13) but at a much lower relative abundance (35%) than in connective tissue. Binding of ^{125}I -IGF1 to muscle fibre in the older fasted lambs was comparable with binding to connective tissue (100%) and in fed lambs was substantially greater (185%). By eight months of age the binding to fibre was absent (0%). These data suggest that an ontogenetic change in the expression of Type 1 receptors may have taken place. Alexandrides *et al.* (1989) measured with radiolabelled hormones the developmental expression of binding of IGF1, IGF2 and insulin to skeletal muscle membranes from rats. They concluded that the induction of specific binding for each hormone was distinct in the ontogenetic timing, binding capacity and pattern of expression. Their results were based on a combination of binding to the various tissues within muscle, not individual tissues as in the current study. The autoradiography data in the present study suggest that ovine Type 1 receptors for individual tissue components of skeletal muscle may also be differentially regulated during post-natal growth.

While ontogenetic development of GH receptors in ovine liver has been reported (Gluckman *et al.*, 1983) there have been no reports of developmental changes in ovine IGF receptors. The possibility remains that the observed changes are not due solely to age but are influenced by two other experimental variables, sex and muscle type. The eight month old sheep used in the experiment reported in Chapter 5 were castrated males and their muscle samples from *M. semi membranosus*. Like the animals in this experiment, the newborn lambs were female, with muscle samples from the *M. biceps femoris*. Possibly, a difference in receptor status exists between these two skeletal muscles of the hind limb.

There was no change in the specific binding of ^{125}I -IGF1 to muscle fibre with fasting in the present study, but binding to the connective tissue was approximately doubled. Increased ^{125}I -IGF1 binding with fasting, ranging from 0 to 250%, has been reported for several rat tissues and the changes determined to be due to alteration of number and

affinity of Type 1 receptors (Lowe *et al.*, 1989). The increase in Type 1 receptors as a result of fasting, both between tissues (Lowe *et al.*, 1989) and between cell types within a tissue, is clearly a coordinated response to nutrient deprivation. Lowe *et al.* (1989) suggest that tissue responsiveness to fasting may depend on whether preservation of a specialised function is required, given that IGFs have a range of metabolic and mitogenic effects on cells. The autoradiography data indicate that the maintenance of functional connective tissue requires a cellular response to substrate limitation and/or the hormonal environment. This may be achieved through regulation of the Type 1 receptor. In cultured myoblasts, Type 1 receptors are down-regulated in number by exposure to IGF1 and IGF2 (de Vroede *et al.*, 1984; Dodson *et al.*, 1987). If the same principle applies *in vivo* this may contribute to a reduced number of Type 1 receptors in well fed animals. The reduced plasma concentrations of IGF1 and lack of immunodetection of IGF1 in muscle from fasted animals (Table 6.21) both suggest that muscle exposure to IGF1 was restricted in this experiment, a possible consequence being the greater expression of Type 1 receptors in connective tissue.

A simpler interpretation of receptor differences between fed and fasted animals is that it may arise from reduced occupancy of a comparable number of receptors *i.e.* with fasting, less endogenous hormone being available to a cell leaves a greater number of unoccupied receptors which are then detected with radiolabelled hormone. This has been considered by Freemark *et al.* (1990) in their investigations of regulation of the placental lactogen receptor by fasting. They found that the specific binding in liver preparations from ewes was greater with fasting, whether or not the membranes were treated with magnesium chloride to remove endogenously bound hormone. Additionally, Lowe *et al.* (1989) reported increased mRNA of the Type 1 receptor in some fasted tissues, suggesting a positive response by cells to increase the number of receptors.

While the competition for ^{125}I -IGF1 binding to connective tissue by 10 ng/ml insulin was not significant, the difference between total and residual binding was greater for fasted

than fed animals. Insulin receptors in skeletal muscle increase in number when animals are restricted in nutrition (Grizard, 1983) or fasted (Contreras, Dohm, Abdallah *et al.*, 1990). Possibly the grain counts reflect a small degree of ^{125}I -IGF1 cross-binding to the insulin receptor in connective tissue of skeletal muscle.

Binding of ^{125}I -IGF2 was restricted to connective tissue and not affected by fasting of the animals (Table 6.15). None of the heterologous unlabelled hormones were effective competitors for ^{125}I -IGF2 binding (Table 6.16, Table 6.17, Table 6.18) and it was concluded that binding was to the Type 2 receptor. The general assumption of limited and approximately equal affinity of each IGF for the heterologous IGF receptor (Van Wyk *et al.*, 1988) does not apply under these incubation conditions and the cell types being studied. In a comparison of competitive potency, IGF2 was much more effective at competing for ^{125}I -IGF1 binding (Table 6.6, Table 6.10) than the reverse (Table 6.16). Others have reported substantially greater potency of IGF2 for ^{125}I -IGF1 binding (de Vroede *et al.*, 1984; Alexandrides *et al.*, 1989; Slootweg *et al.*, 1990) and there has been one observation of IGF2 being more competitive than IGF1 for ^{125}I -IGF1 binding (Duclos and Goddard, 1990). Together with the autoradiography data these reports suggest that IGF2 has a greater affinity for the Type 1 receptor than IGF1 has for the Type 2 receptor.

The autoradiographic localisation of ^{125}I -IGF2 binding to connective tissue is supported by the report of ^{125}I -IGF2 binding to cultured fibroblasts (Thorsson, Hintz, Enberg *et al.*, 1985) and localisation of the Type 2 receptor immunohistochemically to muscle fibre sheaths and perimysium in rats (Valentino *et al.*, 1988). In contrast, Type 2 receptors have been identified on cultured myoblasts (de Vroede *et al.*, 1984; Dodson *et al.*, 1987) and a faint immunostaining of muscle fibre cytoplasm has been observed (Valentino *et al.*, 1988). However, no receptors were identified on muscle fibre in the current experiment. As postulated earlier (p 205) the presence of Type 2 receptors may also be developmentally regulated and, if present in low abundance, possibly be beyond the

sensitivity of autoradiographic detection. Autoradiography has previously been used to localise Type 2 receptors in the brain (Lesniak *et al.*, 1988; Smith *et al.*, 1988) where they are relatively abundant compared with the apparently restricted distribution in muscle. Alexandrides *et al.* (1989) did identify Type 2 receptors in rat skeletal muscle at various developmental ages through to adulthood, but the cellular localisation remains unknown.

^{125}I -IGF2 binding to connective tissue was not altered by fasting. This suggests that the control of Type 2 receptors is relatively insensitive to substrate limitation and the associated hormonal consequences. Other studies *in vitro* arrived at similar conclusions. ^{125}I -IGF2 binding to cultured cells is not altered by pre-treatment with IGF1 or IGF2 (de Vroede *et al.*, 1984; Thorsson *et al.*, 1985). Pre-treatment of cells with GH does not alter binding (Thorsson *et al.*, 1985) but insulin up-regulates numbers of Type 2 receptors (Sessions *et al.*, 1987). The stability of populations of Type 2 receptors is also indicated by steady state internalisation and recycling (Oka and Czech, 1986), highly reproducible specific binding (Thorsson *et al.*, 1985) and binding independent of incubation temperature (Blanchard, Barenton, Sullivan *et al.*, 1988) in cultured cell systems. Thorsson *et al.* (1985) noted an increase in ^{125}I -IGF2 binding with serum starvation of cultured fibroblasts which, if anything, is in contrast to the autoradiography data which suggest that binding might be reduced in fasted animals.

Like binding of ^{125}I -IGF1, the binding of ^{125}I -IGF2 was greatest to blood vessels. In some sections there was greater binding to venule than arteriole endothelium. The presence of Type 2 receptors in vascular tissue has been demonstrated immunohistochemically (Valentino *et al.*, 1988) and IGF binding proteins are secreted by cultured vascular tissue (Bar *et al.*, 1989). In the current study there was no indication from the autoradiographs that unlabelled IGF1 was an effective competitor for ^{125}I -IGF2 binding. There are three types of ovine binding protein, of which one has a greater affinity for IGF2 than IGF1 (Butler and Gluckman, 1986; Hodgkinson *et al.*, 1989a). Unless there was an almost exclusive presence of only the IGF2-specific binding protein

in blood vessels, then binding to binding proteins would have been reduced by co-incubation with unlabelled IGF1. Observations in the current experiment and the experiment described in Chapter 5 lead to the conclusion that the residual binding of IGFs is to the Type 2 receptor.

In connective tissue *in situ* there appears to be a predominance of Type 2 over Type 1 receptors as seen with other tissues and cells (skeletal muscle, Alexandrides *et al.*, 1989; cultured chondrocytes, Sessions *et al.*, 1987; erythroleukaemic cells, Blanchard *et al.*, 1988). The grain counts do not reflect this, but relative exposure times for the emulsion autoradiographs and the image intensities of XAR autoradiographs indicate an abundance of Type 2 receptors. A comparison of emulsion autoradiographs also revealed a qualitative difference in hormone binding to Type 1 and Type 2 receptors. In large regions of connective tissue composed of a significant proportion of matrix, ^{125}I -IGF2 was bound to a greater extent and much more tightly localised to the cell nucleus than was ^{125}I -IGF1 in the same areas. It is possible that the two receptors are differentially localised within the cell in specialised connective tissue, although there are no reports of a nuclear Type 2 receptor. Hartshorn *et al.* (1989) have reported that the predominant location of hepatic Type 2 receptors was to intracellular membranes, which supports the interpretation of the observation made in the current experiment.

CONCLUSION

The physiological response of lambs to prolonged fasting must be comprised of the repartitioning of nutrients to maintain nutrient availability for essential functions and tissues and direction of nutrients from non-essential processes. The plasma concentrations of hormones normally considered to be central in growth processes have been measured in this experiment, in response to fasting. Receptor populations in skeletal muscle have also been measured.

GH concentrations were elevated by fasting, in association with a decrease in plasma IGF1 and no change in IGF2 levels. It is proposed that GH and IGF1 in a fasted state are primarily involved in metabolic processes related to homeostasis rather than promotion of somatic growth. For GH this could be through enhanced actions on adipose tissue, and reduced concentrations of IGF1 have been suggested to be permissive in tissue catabolism (O'Sullivan *et al.*, 1989). Whatever the metabolic reaction of IGF2 to fasting, this was not reflected by a change in plasma concentrations.

In addition to changes of hormone concentrations in plasma, receptor differences in skeletal muscle were also observed. GH receptors available for hormone binding were few in number or absent, suggesting that this tissue is not a target for direct GH action. Type 1 receptors were localised in muscle fibre and connective tissue, but only differed with fasting in the connective tissue. While there is no doubt that fasting has major consequences for skeletal muscle it may be that the mechanisms of atrophy of muscle fibre are not mediated through the GH-IGF axis. It would be interesting to investigate whether hypertrophy of muscle fibres is also independent of changes in Type 1 receptors. In contrast, the functions of connective tissue as a support organ for muscle fibre during hypertrophy and atrophy may well be controlled by IGF1 and Type 1 receptors. In line with the stability of plasma IGF2, Type 2 receptors in connective tissue were abundant but did not alter with fasting. The function of Type 2 receptors in skeletal muscle is as yet undetermined.

In conclusion, these data suggest that the GH-IGF axis is involved with the physiological adaptation of lambs to fasting, predominantly through metabolic actions of GH and IGF1 and the role of connective tissue in the atrophy of muscle fibre.

CHAPTER SEVEN:

GENERAL DISCUSSION

MICROAUTORADIOGRAPHY

The technique of microautoradiography has been developed for use with ovine skeletal muscle throughout the experiments described in this thesis. It has proven to be a sensitive measure of receptors in a target tissue which has been regarded as showing relatively little or no specific binding of IGF1 when this is assessed by conventional biochemical assay (Lowe *et al.*, 1989). A feature of autoradiographic analysis is the small amounts of tissue and radiolabelled hormone from which quantitative measurements of specific binding may be made, approximately 200 µg of protein and 300 cpm of ¹²⁵I-IGF1 per section.

Data in Chapter 5 have indicated that the presence of binding sites for IGF1 was restricted in location to connective tissue and blood vessels which together constitute only a small percentage of the area of a muscle section. The limited distribution of unoccupied receptors within muscle is likely to be largely responsible for the difficulty in demonstrating binding with use of a homogenisation assay. Using the competitive potency of unlabelled insulin for IGF binding as a means of receptor characterisation, the binding sites in skeletal muscle were categorised as Type 1 receptors, Type 2 receptors, insulin receptors or IGF specific binding proteins. Characterisation of binding on the basis of tissue type within skeletal muscle revealed that the IGF1 binding in connective tissue was solely to Type 1 receptors and predominantly to a different binding site in vascular tissue. Potentially, this may have been a Type 2 receptor or IGF binding protein, both of which are present in vascular tissue (King *et al.*, 1985; Bar *et al.*, 1989). Alternatively, a second Type 1 receptor, possibly with higher affinity than that in

connective tissue, may also exist. Type 1 receptors with different affinities for IGF1 (Tollefsen *et al.*, 1987) and tissue-specific Type 1 receptors (Alexandrides and Smith, 1989) have both been reported previously.

Unintentionally, the use of autoradiography has also revealed distinct changes in binding sites for IGF1 which may be a consequence of ontogeny. Control animals in the experiment described in Chapter 5, approximately nine months old at slaughter, had no binding sites for IGF1 in muscle fibre. In contrast, younger control animals used in the experiment described in Chapter 6, six months old at slaughter, showed binding sites for IGF1 in muscle fibre. At some time between these two ages, muscle fibre may have lost the potential to bind IGF1 *in vitro*. Membrane preparations of skeletal muscle from rats also show a post-natal decline of IGF1 binding sites (Alexandrides *et al.*, 1989), but tissue specificity of the changes was not reported.

The final characteristic of IGF binding which was revealed by this autoradiography study is that the distribution and characteristics of binding markedly differ for IGF1 and IGF2. At six months old, binding sites for IGF2 were restricted to connective tissue and blood vessels suggesting, as in other developmental studies of the IGFs (Beck *et al.*, 1988; Han *et al.*, 1988), that the regulation of receptors is not necessarily coordinated for IGF1 and IGF2. These data also substantiated the assumption that receptor cross-binding in studies using tracer quantities of radiolabelled hormone is unlikely if the homologous receptor is present. When used for receptor characterisation, the unlabelled IGFs also showed differing potencies in competing for binding of the heterologous ^{125}I -IGF, IGF2 being a more effective competitor for IGF1 binding to connective tissue than the reverse. Similar observations have been made by others using IGF binding systems *in vitro* (de Vroede *et al.*, 1984; Slootweg *et al.*, 1990).

Qualitatively, macroautoradiographs indicated that the extent of IGF2 binding to connective tissue was greater than of IGF1 binding. This could not be further

substantiated by grain counting as the exposure periods, and therefore grain development, differed between the two incubations. IGF2 binding also showed much stronger nuclear localisation of binding than IGF1 and was greater to venule endothelium than to arterioles.

Technically, the use of microautoradiography does have drawbacks. The production of autoradiographs and grain counting are labour-intensive and time-consuming procedures. This limits the number of incubations which can be undertaken or restricts work to the use of tissue from some but not all animals in an experiment, as for the experiment described in Chapter 6. At present, a reliable and immediate predictor of specific binding from sections like skeletal muscle, which show relatively low levels of binding, has not been found.

Characteristics of the tissue or radiolabelled hormone being used are also important determinants of successful autoradiography. Binding of GH was not demonstrated despite the reported presence of GH receptor immunoreactivity in skeletal muscle (Lobie *et al.*, 1990a). This may have arisen as a result of several factors. The number of GH receptors in skeletal muscle may be low, restricted in distribution, or fully occupied by endogenous GH. Radiolabelled GH also characteristically showed greater levels of non-specific binding than did either of the radiolabelled IGFs, and the non-specific binding of GH was greater to muscle fibre than to connective tissue. Additionally, although satisfactory microautoradiographs were produced with muscle sections, brown pigment prevented them from being produced with liver sections.

The use of a radioiodinated antibody for immunodetection of IGF1 was only marginally successful. The lack of localised antibody binding to blood vessels, which is in contrast to other reports (Hansson *et al.*, 1987; Glimm *et al.*, 1988), indicates that further work is required to optimise the incubation conditions for antibody binding to histological sections. This might utilise an enzyme-labelled antibody, enabling a more immediate

assessment of the extent of immunodetection under various incubation conditions, before progressing to immunodetection with a radiolabelled antibody for quantitation of binding.

EFFECTS OF HORMONE TREATMENT ON GROWTH

The development of prepubertal lambs was altered by treatment with exogenous GH but not GRH or TRH. In the experiment described in Chapter 5, GH did not stimulate weight gain but did affect carcass composition by depressing fatness and elevating water content. These effects have been observed by others (Johnsson *et al.*, 1985; Bass *et al.*, 1991a). In addition to the elevated concentrations of GH in plasma, IGF1 levels were increased. The reduced fatness of carcasses from GH-treated animals is a likely consequence of the anti-insulin-like action of GH on adipose tissue (Goodman, 1981) as IGFs are insulin-like and therefore lipogenic in their metabolic action on adipose tissue (Zapf *et al.*, 1978), so elevated concentrations of IGFs in plasma are incompatible with a reduction of carcass fat.

Several points of evidence suggest that the change in water content of the carcass, as a reflection of the status of skeletal muscle, was not mediated by IGF1. Firstly, there were no Type 1 receptors located on muscle fibre. The possibility that they may have been fully occupied by endogenous hormone is unlikely, considering that neither of the animal treatments (nutritional or GH) which resulted in widely differing IGF1 levels, indicated the presence of a Type 1 receptor in muscle fibre. The relative concentrations of Type 1 receptors in connective tissue did not change with the GH treatment of animals, further suggesting that there was not an increased occupancy by endogenous IGF1. Finally, it has been demonstrated *in vitro* and *in vivo* that IGF1 binding may be regulated by prior IGF1 occupancy of receptors (Rosenfeld and Dollar, 1982; Venkatesan and Davidson, 1990). If locally produced or plasma IGF1 in skeletal muscle was instrumental in altering carcass composition there is every probability that a coordinated change in Type 1 receptors would have taken place.

As GH binding to skeletal muscle was not demonstrated it was not possible to determine whether there was a direct interaction of GH to change water content, or whether there was an indirect effect of GH on a hormone other than IGF1. GH treatment of ovine satellite cells in culture does not alter protein metabolism (Harper *et al.*, 1987; Roe *et al.*, 1989) which supports the carcass composition data, but increased protein content with GH treatment of lambs has been reported by others (Pell and Bates, 1987).

Treatment of animals with GRH might have been expected to cause changes in lamb growth comparable to GH treatment, GRH being the primary stimulating hormone of GH, but this was not the case. Results from the experiment described in Chapter 4 show that the elevation of GH in plasma which was achieved by GRH treatment did not alter carcass composition and was approximately ten-fold less, on a ng/ml basis, than the assayable dose of GH which was effective in the experiment described in Chapter 5. Possibly the dose of GRH which was used, combined with the route of administration or the age of the lambs was not optimal to achieve the extent of GH required to alter growth. In sheep, several weeks of GRH treatment from birth alters some parameters of growth (Pastoureau *et al.*, 1989) and intravenous injection of GRH gives a greater GH response than subcutaneous injection (Della-Fera *et al.*, 1986b).

The evidence of TRH acting as a growth promotant, alone or additively with GRH, was limited to small gains in body weight later during the experiment described in Chapter 4. A synergistic action of TRH treatment on GRH-stimulated GH secretion was suggested, but not significant, contrasting with data *in vivo* and *in vitro* from cattle (Brazeau *et al.*, 1986; Hart *et al.*, 1988). Collectively, the data from Chapter 4 indicate that the small stimulation of growth by TRH, independently or additively with GRH, was unlikely to be mediated by the GH-IGF axis.

EFFECTS OF NUTRITION ON GROWTH

Variable nutrition affected growth dramatically. Prolonged fasting or restricted intake of food reduced the body, carcass and organ weights of prepubertal lambs when compared with well-nourished animals. Carcass composition was also altered in a manner whereby the carcass components remained constant as a proportion of carcass weight, unlike the more selective effects of GH on carcass composition. This reflects the integrated response of the animal to nutritional stress, allowing subsistence and survival. Nutrients, whether mobilised from muscle and fat by catabolism and lipolysis respectively, or ingested, were directed from growth to more essential homeostatic processes.

GH concentrations were elevated with fasting, as reported by others (Driver and Forbes, 1981), but not with poor nutrition. Plasma levels of IGF1 were decreased at both levels of nutritional stress, which is also in agreement with other studies (Elsasser *et al.*, 1989; Freemark *et al.*, 1990). Data in this thesis suggests that IGF1 concentrations in sheep may be a more sensitive indicator of nutritional status than GH, which contrasts with the conclusion made by Breier *et al.* (1988b) for cattle. There is also the possibility that a differential sensitivity between GH and IGF1, to changes in nutritional status, arose as a consequence of different ages of the animals in the experiments described in Chapters 5 and 6. There may be decreased sensitivity of GH in particular to nutritional manipulation in older lambs.

The detection of IGF1 in skeletal muscle reflected the relative plasma concentrations of IGF1 in the fasted and fed groups. This may have resulted from IGF1 transport from plasma (Hodgkinson *et al.*, 1990) or represent the secretion of IGF1 from skeletal muscle itself (Jennische *et al.*, 1987). It is unlikely to be a measure of endogenous hormone bound to Type 1 receptors in the connective tissue of fed animals, resulting in reduced binding capacity for ^{125}I -IGF1 *in vitro*. The detection of IGF1 was only

significant when the data for connective tissue and fibre were combined and there was no evidence of localised binding of the antibody to connective tissue.

The lack of a change in binding of IGF1 to fibre with fasting and the failure to demonstrate Type 1 receptors with poor nutrition strongly suggests that IGF1 played no part in the adaptation of muscle fibre to nutritional stress. The opportunity for IGF1 to act as a mitogen in fully differentiated muscle fibre is probably limited to replication of satellite cells to provide myonuclei as required when fibres hypertrophy, as hyperplasia is completed before birth in sheep (Sivachelvan and Davies, 1986). Satellite cells are difficult to identify from myonuclei with the light microscope, and constitute only a few percent of the total nuclei population (Snow, 1977), so changes in Type 1 receptors at this level would not be detected by microautoradiography.

IGF1, as an insulin-like molecule, is considerably less potent in its anabolic action on cells than insulin (Poggi *et al.*, 1979). O'Sullivan *et al.* (1989) have proposed that reduced IGF1 concentrations in plasma during fasting may be permissive in allowing tissue catabolism to take place. Data from the experiment described in Chapter 6 would suggest that if this is so, the anabolic and catabolic effects of IGF1 were not mediated by binding to the Type 1 receptor in muscle fibre. There was an indication that the insulin receptor in fibre increased in binding capacity with fasting, as shown by a seventeen-fold difference in grain counts between fed and fasted animals. This was not a significant effect, but implicates the insulin receptor as being regulated by nutrition, as demonstrated by Grizard (1983) in muscle membranes.

The role of connective tissue in skeletal muscle is such that it expands and contracts in concert with hypertrophy and atrophy of the muscle fibre. The increase in Type 1 receptors with fasting and continued presence of Type 1 receptors in older lambs, and the presence of Type 2 receptors suggest that connective tissue is a more sensitive target tissue for nutritional manipulation through the GH-IGF axis than is muscle fibre. As

considered earlier, the increased binding is unlikely to be a consequence of reduced occupancy by endogenous hormone. Increases in IGF1 binding and mRNA for the Type 1 receptors have been reported for a number of tissues in fasted rats (Lowe *et al.*, 1989), suggesting that a positive response of receptor synthesis has been initiated. There is more likelihood in connective tissue than in muscle fibre for a mitogenic action of IGF1 in conjunction with hypertrophy or atrophy of the fibre, but this is difficult to reconcile with a concurrent increase in receptor number and decrease in IGF1 secretion.

CONCLUSION

Hormonal and nutritional manipulation of prepubertal lambs has resulted in changes in growth and carcass development. This does not appear to have been strongly linked with effects of the GH-IGF axis in skeletal muscle. Levels of IGF1 in plasma were manipulated by nutritional or GH treatment without difficulty, but changes in growth were small and took place without concurrent changes in the receptor populations for IGF1 in muscle fibre. Instead, connective tissue appears to be a target tissue through which IGF1 may mediate the effects of nutritional status. Ontogenesis may have been a significant factor in the sensitivity of the GH-IGF axis to experimental manipulation, and similar work with younger lambs could have significantly greater impact on their growth.

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