Metabolic control of wool growth in Romney sheep

A thesis
submitted in fulfilment
of the requirements for the degree of
Doctor of Philosophy

by
Lucretia Henriette Dijkstra

Lincoln University
1994

Abstract

Wool strength is a heritable trait and varies widely between individuals even when they are maintained under equivalent conditions nutritionally. Consequently, it is valid to search for genetic markers of wool strength with the aim of using them in improving fleece quality. Wool strength is closely related to winter wool growth (Geenty *et al.* 1984). It is thought that the diminished follicle activity at this time reduces fibre diameter (Ross *et al.* 1965). This thesis investigates some of the mechanisms which may control wool growth. Such understanding will consequently aid a future search for genetic markers of wool strength.

Blood plasma from eighty-six sheep was assayed for a number of metabolites and growth factors including glucose, urea, β-hydroxybutyrate, creatinine, insulin, insulin-like growth factor I, growth hormone, cortisol and melatonin. A correlation was observed between elevated plasma insulin and reduced plasma glucose and wool strength. Some changes in peripheral tissue sensitivity to insulin were also found. For example, in an indoor treatment high wool strength animals showed some peripheral tissue resistance to insulin. These data suggest that the control of and/or mechanisms of glucose uptake vary between high and low wool strength animals.

Insulin binding by the skin was investigated. No significant differences in insulin receptor binding were found between the skin of high and low wool strength animals. Although it is possible that differences could have been masked by the variable nature of the data, the evidence available from this and previous studies suggest that highly variable insulin binding between individuals by ovine tissues is a 'normal' observation. The lack of correlation between insulin receptor binding and wool strength was consequently thought to be real. Differences in tissue sensitivity to insulin were therefore hypothesised to lie with a post-receptor mechanism.

The expression of glucose transporters 1 and 4 was assessed in tissues of high and low wool strength sheep using human cDNA probes. Appropriate human cDNA probes were also used to evaluate the expression of the insulin and insulin-like growth factor I receptor genes and the gene for ribosomal protein S6. Except for the ribosomal protein S6 gene, low to nil expression of these genes was observed in all the tissues examined when compared to the constitutively expressed B-actin gene. Expression could not be stimulated by chronic (5 hour) insulin infusion. Low expression was not thought to be due solely to a lack of homology between ovine and human sequences suggesting the expression of genes involved in glucose uptake is extremely low in sheep.

The glucose transporter I gene was expressed at low levels in skin, wool follicles and brain tissue but not in muscle and fat tissue. This suggests that skin, like brain tissue, has an absolute requirement for glucose, a situation not found in adipose and muscle tissue. Glucose transporter I is generally reported to be associated with non-insulin dependent glucose uptake. From this it was concluded that elucidation of the mechanisms by which glucose is taken up into different tissues provides the key to understanding the controlling factors by which glucose is partitioned to the wool follicle. Such a mechanism could be influenced by insulin either directly by its action on the wool follicle, or indirectly, by causing insulin resistance in muscle and adipose tissues which partitions glucose to the skin where it is taken up in an insulin-independent manner.

Acknowledgements

Completion of this thesis would have been extremely difficult without the help of my supervisors, family and friends. With this in mind, I would like to thank Dr Roy Bickerstaffe for his continual support and valuable critique regarding the interpretation of results and in the design of the trials. A big thanks must also extended to Dr Jon Hickford, who taught me the practical skills necessary for the molecular biology sections of this work, as well as thoroughly reading this thesis which resulted in many helpful suggestions.

The field work would not have been possible without the support of AgResearch Lincoln via Dr Andy Bray, who provided the necessary animals and facilities as well as some very capable and amiable staff including Malcolm Smith, Denis O'Connol, Norma Merrick, Phil Barnes and David Baird. Thank you for the fun times and the hard work. Furthermore, Dr Patricia Harris of AgResearch Palmerston North was incredibly generous with her hospitality, time and facilities in performing the insulin infusion experiment. The opportunity proved to be a very positive learning experience for me - both personally and academically.

In 1991 the Vernon Willey Trust sponsored my visit to Australia to visit scientists at the University of Adelaide, Melbourne University and CSIRO at Prospect, Sydney. This allowed me to discuss my thesis proposal extensively with those experienced in the field and gave me many ideas for future research. I would like to hereby thank the Trust for this valuable opportunity.

There are many people within the department who have been instrumental in providing a pleasant, warm and stimulating work environment. Dr. Sami Damak, Tania Gourley, Peter Isherwood, Anne Hill, Chris Dawson and Jane Brennan were always quick to help out and provide helpful suggestions as well as being absolute lifesavers at times! Sue Leslie and Anne Rose - thank you for being my buddies and for your sense of humour. I would also like to thank Dr Sue Mason for being the friendly and warm person that she is. I have shared many good and bad times with those I have shared a lab with over the years. These budding scientists have provided an essential support network and hence I would like to thank Dr Sue Francis, Dr Jinny Willis, Geraldine Rogers, Jo Harrison, Mary-Clare Craigen, Matthew Kent, Christine Couldrey, Andrew Cridge and Leila Markham.

I have often wondered what made me strive to complete a Ph. D. My friend Dr Martin Bell has been a real inspiration at times and was responsible for cutting some good steps to follow up the mountain. Similarly, Dr Alan Wood, being a fellow sufferer of the Ph. D. affliction, has been a great support on several occasions. But most of all I believe the faith, love and confidence I have received from my family were responsible for keeping me sane when I felt the hurdles were insurmountable - this is your thesis too.

and every hour of every day i'm learning more
the more i learn the less i know about before
the less i know, the more i want to look around
digging deep for clues on higher ground.....

UB40
Promises and Lies, 1993

Table of contents

List of tables

List of figures

Seasonal wool growth and wool strength

Wool growth in the New Zealand Romney is modulated in response to season. Winter growth can be half that of summer growth (Geenty *et al.*, 1984, Hawker and Crosbie, 1985). Reduction in wool growth is due to changes in fibre diameter and length (Ross, 1965). This results in a narrower region along the fibre called the winter break where most fibres break under tensile stress (Anderson and Cox, 1950).

Fibres of high tensile strength are desirable to the wool industry because low strength wools break during the carding process (mechanical untangling and straightening of fibres before spinning). This results in shorter fibres which decrease the processing performance of wool and thereby reduce the number of end-uses available for the fleece (Rogan, 1989). The consequential annual losses may be as high as $100 million (calculated from 1988/89 NZ Wool Board figures), through wool being downgraded and devalued when end-use becomes limited. Additional disadvantages of low strength wool are associated faults such as cotting and yellowing (Ross, 1982), which further reduce the value of the wool.

Tensile strength of an individual fibre correlates with the tensile strength of a staple (a length of wool which requires twisting and spinning to form a yarn, Hunter *et al.*, 1983 Fitzgerald *et al.*, 1984). Since measuring staple strength is a faster technique than measuring individual fibre strength, it is the method of choice for the measurement of wool strength, both in the industry and within this thesis.

Wool growth and staple strength vary in response to a variety of nutritional, physiological and environmental factors (Corbett, 1979, Bigham *et al.*, 1983, Reis, 1992). However, genetic factors are also likely to be important since staple strength still varies between animals maintained nutritionally, physiologically and environmentally equivalent (Geenty *et al.*, 1984). Genetic variation can result in up to 9-fold differences in staple strength between individual sheep in flocks managed under controlled conditions (Heuer, 1979, Geenty *et al.*, 1984, McClelland *et al.*, 1986). The effect of genotype on wool growth can be convincingly demonstrated by comparing seasonal changes in wool growth between breeds. Such changes result in shedding of the fleece (which equals very low wool strength) in the New Zealand Wiltshire while very little seasonal wool growth exists in the New Zealand Merino.

Under the assumption that wool growth is a function of nutrient supply to the wool follicle, genetic differences in wool strength and staple strength may eventuate at various stages of nutrient flow. For example, differences between animals could arise in the uptake of nutrients from the gut, partitioning of nutrients to the wool follicle, and nutrient uptake by the wool follicle itself. The rate and extent at which these processes occur may not necessarily be constant between animals and across season. For example, Hawker and Crosbie (1985) have shown that the response of wool growth to variation in nutrition is twice as great in summer as in winter. In addition, animals which produce weak wool have a more pronounced difference between winter and summer patterns of wool growth (Hawker, 1984, Hawker and Littlejohn, 1989) suggesting that the relative partitioning of nutrients between tissues may not be equivalent between individuals.

Given the degree to which genotype has been shown to influence the seasonality of wool growth, the search for genetic markers of wool strength with the aim of improving breeding stock, becomes an attractive proposition. Such markers could increase the rate and efficiency of selection for wool strength in breeding programmes. They can also increase the carrying capacity of the farm by allowing the earlier culling of low wool strength animals. The key to increasing wool strength by using genetic markers is likely to lie with greater understanding of the mechanisms which increase wool growth, potentially by controlling nutrient partitioning to the wool follicle. Differences in nutrient partitioning between low and high wool strength animals are likely to be most evident during winter when wool growth differences between low and high wool strength animals are most pronounced (Hawker, 1984, Hawker and Littlejohn, 1989).

1.1 Thesis objective

This thesis commenced with a screening trial which investigated the association of endocrine and metabolic markers with indicators of winter wool growth including minimum fibre diameter and staple strength. Once such a marker was found it was used to limit the ensueing study of wool follicle gene expression to several loci involved in glucose uptake.

Literature review: the biology of wool growth

2.1 Introduction

The research into seasonality of wool growth and tensile strength of wool is not new. Considerable effort has been directed into these areas by the Australian Commonwealth Scientific and Industrial Research Organisation (CSIRO), the South African Wool and Textiles Research Institute (SAWTRI) and the Wool Research Organization of New Zealand (WRONZ). Biological research (reviewed Bigham *et al.*, 1983, Reis, 1992) has focused on the effect of nutrition on the composition and synthesis of wool proteins. However, the biological approaches have generally not been extended to specific wool quality traits such as staple strength, and few of the biochemical and molecular mechanisms controlling wool growth have been defined.

This literature review gives an overview of the biology of wool growth with particular emphasis on wool strength. Its breadth reflects the factors considered in the initial design of the research in this thesis.

2.2 Environmental determinants of staple strength

2.2.1 Season

Weak wool (break or fleece tenderness, Figure 2.1) has been studied mostly in terms of reductions in fibre diameter and wool growth in response to season, since winter may reduce fibre diameter by as much as 40% (Story and Ross, 1960). It is not clear what season dependent environmental factor is responsible for decreases in winter wool growth. There is evidence to suggest that both exposure to low temperature, and photoperiod play a role, as well as seasonality in feed intake (see section 2.2.3).

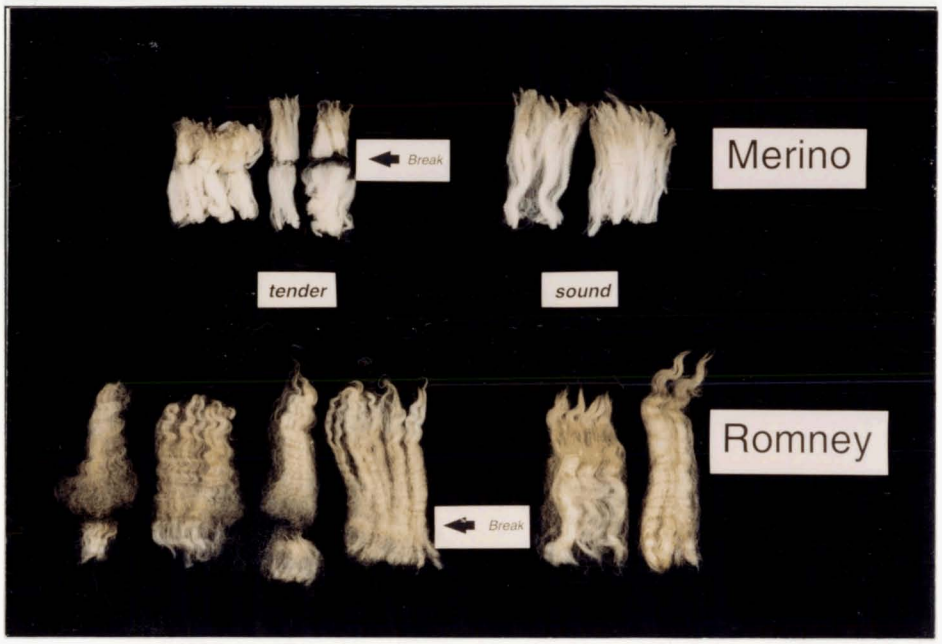


Figure 2.1: Wool break.

Examples of tender (weak) and sound (strong) staples for Romney and Merino fleeces are shown. Tender staples have been pulled apart to demonstrate the break region.

Evidence for the effect of low temperature on wool growth is ambiguous. Downes and Hutchinson (1969), studied the effect of low temperature on wool growth by exposing two sheep to ambient temperatures of 2 °C for four days. They found that the average length of wool grown by a clipped skin patch was reduced by 30% per day. The effect on diameter was unclear. Doney and Griffiths (1967) and Lyne *et al.* (1970) reported that diameter does not vary in response to low temperature and confirmed that wool growth is reduced. However the validity of experiments such as this which utilized clipped areas of skin to measure wool growth has been questioned. Laboratory studies have shown that the rate of wool growth on clipped patches differs from growth rates of unclipped areas (reviewed, Bottomley, 1989). Bottomley (1989) therefore suggests that wool production of well-fleeced sheep is not influenced directly by temperature.

Hart (1961) and Hart *et al.* (1963) have demonstrated that wool growth can be manipulated by changing photoperiodic rhythms. Hart (1961) reported that changes in fibre length are associated with a change to a "summer" light regime but no simultaneous changes in fibre diameter were reported. In contrast, a study of goats (McDonald and Hoey, 1987) suggested that photo-translocation (a change in day:night light ratio) significantly affected

both fibre diameter and fibre volume. Furthermore, rhythms in fibre volume growth rate of cashmere and hair follicles were not equivalent (McDonald *et al.*, 1987) suggesting follicle specific mechanisms mediate the effects of photoperiod.

2.2.2 Pregnancy and Lactation

Wool production in the breeding ewe is reduced 10-14% by the demands of pregnancy and lactation (Corbett, 1979, Oddy, 1985). This is reflected in reductions in fibre diameter and fibre length (Brown *et al.*, 1966). However, seasonal reductions in fibre diameter cannot be attributed solely to pregnancy. This is because seasonal variation in fibre diameter is observed both in rams (this thesis), and dry ewes (Geenty *et al.*, 1984).

The effect of pregnancy on winter wool growth and staple strength can, in part, be alleviated by extra feed, particularly during mid-pregnancy (Fitzgerald *et al.*, 1984, Williams and Butt, 1989). This period coincides with the time that minimum fibre diameters are generally observed in New Zealand (July-August, Hawker and Littlejohn, 1989). It is probable that the observed changes in wool growth during pregnancy are due to the combined effects of photoperiod and an increase in partitioning of nutrients from the ewe to the foetus.

2.2.3 Nutrition

Greater feed intake increases winter wool growth (Hawker and Crosbie, 1985, Sumner, 1983, Rowe *et al.*, 1989) and fibre diameter (Sumner, 1983 Rowe *et al.*, 1989). Black *et al.*, (1973), showed that both protein and energy supply have the potential to modulate wool growth. The relative importance of these two components was shown to be dependent on nutritional state before protein or energy supplementation. Beneficial effects of protein on wool growth were also found to occur by Saul and Channon (1990) when sheep fed a protein rich legume diet *ad lib* grew 34-39% more wool than sheep fed grass hay.

Hemsley and Reis (1985) concluded that the major factors influencing the amount and characteristics of wool grown are genotype and nutrient supply. This is because although nutrition was found to affect wool growth, differences in wool growth between lines of sheep with similar live weight but different fleece weight could not be directly attributed to intake (McClelland *et al.*, 1986). Furthermore, Bigham *et al.*, (1983) have clearly shown

that seasonal wool growth still occurs if intake is constant. It is likely however that seasonality of wool growth is aggravated in the normal farming situation by reductions in feed intake and quality which are commonly observed in winter.

2.2.3.1 Amino acid intake

It may be that all amino acids are required for wool growth. However the omission of some is particularly severe, while the inclusion of others is particularly beneficial. Abomasum infusion experiments have shown that only the sulphur amino acids stimulate wool growth (Reis and Schinkel 1962, 1964, Reis *et al.*, 1973, Reis, 1979). Methionine and cysteine are important components of wool proteins as shown by the sulphur content of wool which varies from 2.7-4.2% (Reis, 1965). Most of this sulphur is present as cystine or cysteine (97%) with the remainder provided by methionine (Reis and Schinckel 1964). Omission of methionine from an otherwise complete mixture of essential amino acids, inhibits wool growth and reduces the tensile strength of wool (Reis and Tunks, 1978). Its benefits to wool growth may only be effective if animals are fed below maintenance nutrition (Stephenson *et al.*, 1991). High levels of methionine are inhibitory to wool growth and this inhibition is independent of food intake or body weight (Reis, 1967).

Interestingly, equimolar amounts of methionine can replace the requirement for cysteine (Reis, 1979). This may in part be because methionine converts to cysteine via a trans-sulphuration pathway (Hemsley and Reis, 1985). Apart from providing a substrate for trans-sulphuration, methionine itself also appears to play an essential role in stimulating wool growth (Reis and Tunks, 1978). This is because wool growth responds more to abomasal infusions of methionine than infusion of equivalent amounts of cysteine (Williams *et al.*, 1972). Methionine is a precursor of the methyl group donor S-adenosyl methionine (SAM), and N-formylmethionine (the initiating amino acid in protein synthesis). In addition, decarboxylation of SAM by SAM decarboxylase is a crucial step in polyamine synthesis. Polyamines have been associated with increases in protein and DNA synthesis (Tabor and Tabor, 1976). There is evidence that plane of nutrition significantly increases the activity of SAM decarboxylase in the wool follicle (Jarvis *et al.*, 1990). An interesting hypothesis is that since high wool producing sheep do not appear to have a higher level of available cystine at the wool follicle (Hemsley and Reis, 1985), high wool producers may have a greater capacity to produce SAM in the follicle.

2.2.3.2 Minerals

The dietary supply of many minerals indirectly affects wool properties and growth by influencing rumen metabolism and feed intake. However, Purser (1979) after reviewing the effects of selenium, sodium, potassium, fluorine, cobalt, manganese and phosphorus, concluded that only zinc and copper are likely to have a direct effect, independent of feed intake.

Zinc deficiency weakens wool fibres. It can lead to the complete cessation of fibre growth and partial or complete loss of the fleece. Fibres are degraded and distorted during the deficiency, and much of the fleece is ultimately shed (Mills *et al.*, 1967). In preruminant lambs, zinc deficiency reduces feed intake and live weight gain (Masters *et al.*, 1985). White *et al.*, (1994) suggest that zinc deficiency reduces wool growth by impairing wool protein synthesis and keratinisation.

Copper is a cofactor required for the formation of disulphide bonds (Marston 1949). Copper deficiency induces the growth of "steely wool" of low tensile strength. Fibres lack crimp and are very lustrous (Marston, 1946, Lee, 1956). The sulphur content of the wool is also diminished (Gillespie, 1964).

2.2.3.3 Vitamins

Lambs reared solely on synthetic liquid diets show abnormal wool growth with marked weakening of fibres and alopecia (hair loss). These symptoms are alleviated by supplementation in the B group vitamins, particularly folic acid (Chapman and Black, 1981).

Vitamin A has been shown to increase the cysteine content of hair in rats (Koyanagi and Odagiri, 1960) and children (Koyanagi and Takanohashi, 1960). Its derivative, retinoic acid, has been demonstrated as being involved in the differential expression of keratin intermediary filament genes in skin (Gilfix and Eckert, 1985, Blumenberg *et al.*, 1992). Such differential expression of keratins is an important part of skin development and repair (reviewed Fuchs, 1988).

2.2.4 Weather

Studies on the effect of weather on wool growth have mostly been concerned with temperature stress as a function of air temperature, humidity and wind chill (reviewed Bottomley, 1979). The effect of low temperature on wool growth has been discussed in section 2.2.1. The intrinsic strength of a fleece (fleece tenacity) may also be influenced by the mechanical stresses imposed by weather. The more exposed parts of the body such as the back, have weaker wool (Ross, 1984). In addition, moisture on the belly wool promotes microbial attack which can reduce the intrinsic strength of wool (see section, 2.2.6).

2.2.5 Age

Obvious trends between age and staple strength have not been observed (Ross, 1965, Bigham *et al.*, 1983, Fitzgerald *et al*, 1984). While there is a trend for ewe hoggets to show a higher proportion of tender fleeces over mature ewes (Bigham *et al.*, 1983), this is probably due to the partitioning of nutrients to the foetus and ewe (both of whom have not reached full size), at the expense of the wool follicle.

2.2.6 Ill Health

The effects of parasites and disease may range from small reductions in wool growth to complete shedding of the fleece. Changes may involve variations in fibre diameter and fibre length (Donald, 1979). Infestation by parasites, systemic disease, and gastrointestinal infections affect wool growth by impairing nutrient availability and utilization from the gut (reviewed Donald, 1979). Most effects of disease on wool growth are likely to be dependent on the reduced feed intake associated with illness. As an example, footrot mostly reduces wool growth via the impairment of grazing (Marshall *et al.*, 1991).

Mycotic dermatitis, fleece rot and Bolo disease can all directly affect wool fibres. Bacterial infections of the skin, such as Bolo disease, significantly affect the tensile strength of wool (Colly *et al.*, 1990, Van Tonder *et al.*, 1990).

2.2.7 Time of Shearing

Wool from sheep shorn before lambing (August-September) has staples with winter break regions close to the bottom (skin end) of the staple. The net effect of prelamb shearing therefore, is to increase the length after carding of the wool (Story and Ross, 1959). However prelamb shearing is avoided by many farmers of Cross-bred sheep because of: (1) an increase in feed requirement to maintain body temperature, (2) the cold conditions that can be encountered at this time which result in greater stock losses and (3) it reduces animal suffering by prevention of lacerations caused by the shearing apparatus (Marshall, A., Wools of New Zealand, personal communication, 1994).

2.3 Physiological studies of wool growth

The endocrinology of wool growth has attracted the attention of many researchers. This is because of the large effect that hormones can have on the partitioning of nutrients to different tissues in ruminants (Barry *et al.*, 1982, Guesnet *et al.*, 1991, Harris and Lobley, 1991, McDowell and Annison, 1991, reviewed, Sugden and Fuller, 1991, Bickerstaffe, 1993). Consequently a substantial amount of work demonstrates the role of the endocrine system in the control of wool follicle activity.

2.3.1 Gonadal hormones

Domestic sheep do not exhibit changes in pelage at puberty. However, there is no doubt sex hormones can affect hair follicle activity in some species since many mammals have a juvenile and adult pelage. Gonadal hormone concentrations can show marked seasonal fluctuations and ram testosterone levels show a distinct annual rhythm (Lincoln and Ebling, 1985). Lincoln (1984), demonstrated however, that castration had no effect on seasonal wool growth. Progesterone does not affect wool growth, while oestradiol is inhibitory (Slen and Connell, 1958). The inhibition by oestradiol may occur via stimulation of the adrenal cortex which presumably stimulates cortisol secretion (see section 2.3.6 for the effect of cortisol on wool growth).

Human studies have provided contradictory evidence for the role of androgens in hair growth. For example, Maudelonde *et al.*, 1986, reported that androgens did not directly affect hair cell proliferation or protein synthesis in pubic and scalp hair. More recent

studies however, have shown that regional differences in androgen metabolism can be established in alopecic (bald) and non-alopecic areas of patients with male pattern baldness (Puerto and Mallol, 1990). Furthermore the nature of the relationship between male pattern baldness and androgen levels is unclear. While high androgen levels traditionally have been associated with baldness, Knussman *et al.*, (1992) in fact reported the reverse relationship.

2.3.2 Thyroid hormones

Photoperiod has been shown to affect the activity of the thyroid gland in Soay sheep (Lincoln *et al.*, 1980). However, the effect of thyroid activity on wool growth remains unclear. While, Kirton *et al.* (1959) have shown that administration of thyroxine increases wool growth, other experiments have not confirmed this relationship. For example, plasma thyroxine (T4) and triiodothyronine (T3) levels were not different between high and low fleece weight lines, nor were the responses of these hormones to thyroid releasing hormone (TRH) any different (Sun *et al.*, 1992). Furthermore, Salem *et al.* (1991) concluded that wool growth increased with declining T3 and T4 levels while Wallace (1979) concluded that it was unlikely that thyroid hormones regulated wool growth in normal sheep since 15% of normal plasma T4 levels produce near normal fibres. Hynd (1989) also suggested a more indirect and permissive role for T4 in wool growth.

2.3.3 Insulin and insulin like growth factors

Although the anabolic effects of insulin on tissues such as muscle are well documented, a negligible amount of work has been done on the effects of insulin on skin and wool growth. Interestingly, insulin resistant states in humans have been associated with the condition *Acanthosis nigricans* - a thickening and hardening of the skin (Kahn and Podskalny, 1980, Geffner and Golde, 1988). This implies insulin is required for normal skin function. In sheep, Oddy and Lindsay (1986) have reported a positive correlation between plasma insulin and wool growth.

Insulin like growth factor I (IGF-I) has biological actions very similar to those of insulin in adult male rats it increases peripheral glucose uptake, it increases glycogen synthesis, decreases plasma amino acid levels and presumably decreases the rate of protein degradation (Jacobs *et al.*, 1989). Only one study has been completed regarding the effect

of IGF-I on wool growth where direct intramuscular administration of IGF-I in lambs did not produce any changes (Cottam *et al.*, 1992).

2.3.4 Growth Hormone

In a study with Merino sheep on a high energy plane of nutrition, growth hormone (GH) treatment was associated with increases in plasma insulin and IGF-1 while T_4 and cortisol decreased (Wynn *et al.*, 1988). During treatment wool growth and fibre diameter also decreased. Once GH treatment ceased, wool growth increased by 20% compared to control animals. The hypothesis was put forward that the 20% depression of plasma methionine observed during the treatment had limited wool growth. GH may therefore have induced changes in wool growth by changing the partitioning of amino acids between the wool follicle and other tissues. After GH treatment ceased, homeostatic mechanisms mobilized tissue protein (deposited as the result of treatment) to stimulate wool growth in the manner observed. Although this study has clearly illustrated a negative effect of GH on wool growth, positive effects have been found by other workers (Zainur *et al.*, 1989, and Sun *et al.*, 1992).

2.3.5 Prolactin and melatonin

Some more primitive breeds of sheep, such as the Limousine and Soay, show marked photoperiod dependent reductions in wool growth culminating in a moult, an event which is suppressed by pinealectomy (in Limousine rams, Panaretto *et al.*, unpublished). Melatonin has also been implicated in mediating reproductive responses to photoperiod (Lincoln *et al.*, 1982, Almeida and Lincoln, 1982, Bittman *et al.*, 1983, Lincoln and Ebling, 1985). The biological effects of melatonin could be mediated by prolactin, a hormone usually implicated in the initiation of lactation (reviewed Nicoll, 1974). Evidence for prolactin as a mediator of melatonin action is that plasma prolactin levels are regulated at least in part, by melatonin (Lincoln and Ebling, 1985). Pinealectomized Limousine rams also show different plasma prolactin levels over control rams exposed to the same light regime (Allain *et al.*, 1986). Consequently, prolactin, rather than melatonin was suggested as modulating seasonal wool growth in the Soay (Lincoln and Ebling, 1985, Lincoln, 1989).

It seems plausible that the reduction in wool growth in response to photoperiod in

modern Romney and Merino breeds, is an evolutionary moulting remnant from earlier ancestral breeds. Consequently, a likely candidate to mediate this response is melatonin. However Foldes *et al.* (1990), found that melatonin implants did not suppress monthly patch wool growth or annual wool production in Merinos. Harris *et al.* (1989) obtained similar results with Romneys. In addition, Scott *et al.* (1992) found no difference in day and night melatonin levels between Romney sheep with high or low levels of seasonal wool growth, and Houghton *et al.* (1993) reported that in crossbreeds (such as the Romney), diurnal variation in plasma prolactin is directly under the control of external light-dark cycles, not melatonin. The relationship between photoperiod, melatonin and prolactin is therefore inconsistent between breeds.

2.3.6 Glucocorticoids

Activity of the adrenal cortex is influenced by cold and extreme weather (Reid, 1962, Panaretto and Vickery, 1970) giving it the potential to be particularly active in winter. Indeed, Lindner and Ferguson (1956), in their study of sheep given a daily intramuscular injection of adrenocorticotrophic hormone found that wool growth was significantly decreased and rose sharply with cessation of treatment. Both fibre thickness and length were reduced, which produced a break in the fleece.

The detrimental effect of cortisol on skin metabolism includes complete cessation of wool follicle activity coupled with inhibition of hair growth. Large prolonged doses of cortisol and cortisol analogues, depress mitotic activity and follicle bulb size (Chapman *et al.*, 1982). They also affect the ability of skin to utilize glucose (Chapman and Bassett, 1970). In the case of dexamethasone, it may reduce wool growth directly by the inhibition of DNA synthesis in the wool follicle (Panaretto *et al.*, 1982). There is little evidence however that physiological doses of cortisol affect wool growth (Hynd, 1989). Similarly, Scobie (1992) found that systemic or intradermal injections of cortisol did not change cell division within a day. *In vitro* studies using skin strips also did not show wool follicles to be responsive to cortisol.

2.3.7 Catecholamines

Catecholamines and their well known effect on the central nervous system may regulate wool growth and natural shedding via the partitioning of blood flow. Removal of the

thoracic sympathetic nerve from one side of the neck increased blood flow and enhanced wool growth on the operated side (Ferguson, 1949). The study found that denervation increased fibre volume, by increases in fibre length, not fibre diameter. A direct inhibitory peripheral effect on wool growth has been observed (Cunningham *et al.*, 1979). Intradermal injection of noradrenaline inhibited wool growth via a mechanism involving alpha-adrenergic receptors located in skin. The effect was distinct from the inhibitory effect of noradrenaline on cell division which is mediated by beta-adrenergic receptors. However, the mechanism did not involve vasoconstriction since vasoconstrictors could not mimic the response. Scobie (1992) using skin strips showed that *in vitro* adrenaline and noradrenaline decrease DNA synthesis.

2.3.8 Epidermal growth factor (EGF)

Gillespie *et al.*, (1982) found that jugular infusions of EGF could severely inhibit wool follicle activity to the extent that the whole fleece was shed two weeks after infusion. *In vitro*, EGF directly inhibits follicle activity and fibre growth (Philpott *et al.*, 1990). Interestingly, EGF only appears to reduce fibre diameter and wool growth if administered systemically. Peripheral infusion into the deep circumflex iliac artery has no adverse effects on wool growth, although EGF levels of the efferent vessel were equivalent to levels found in sheep defleeced with systemically administered EGF (McDonald *et al.*, 1983). This suggests that some concurrent endocrine change, or alternatively, a peripheral alteration of EGF molecules, cannot occur if EGF is locally administered. In support of the occurrence of simultaneous changes in endocrine factors, Moore *et al.* (1984) found that increases in plasma growth hormone and placental lactogen concentration occurred in plasma of ewes during EGF treatment. Circulating levels of thyroxine declined. Defleecing of sheep using EGF has been assessed as a means of chemical defleecing (Young and Field, 1988, Panaretto *et al.*, 1989, see section 2.8).

2.4 Wool follicle morphology

Wool follicles are tubular down growths of the epidermis into the dermis. The wool fibre-follicle complex is a complicated structure (figure 2.2), and consists of an active metabolic region (follicle bulb) which anchors the beginnings of the less metabolically active wool fibre consisting of a cuticle, a cortex and in larger fibres, a medulla. The bulb region, encloses a population of highly mitotic cells which are derived from the dermis. These

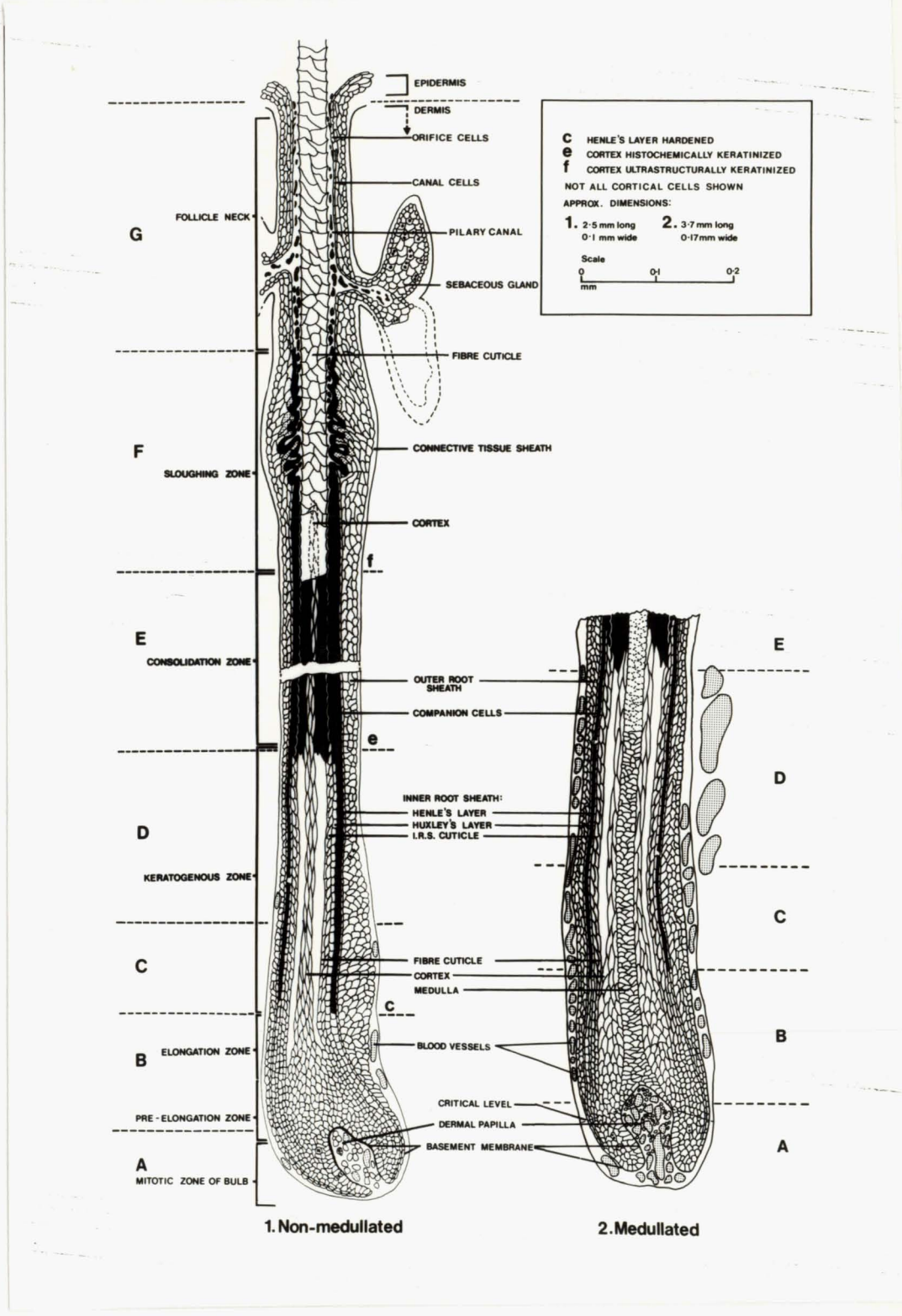


Figure 2.2: Structure of the wool fibre and follicle

Cells are derived from the mitotic zone in the bulb (Zone A) and move up to the follicle before they lose their potential for cell division. In Zone B, differentiation becomes visible as illustrated by cell growth and changes in shape. Keratin synthesis occurs in Zones B, C, and D. Zone E is a region of cross-linking, stabilization and dehydration (Orwin, 1989, with permission).

cells, collectively called the dermal papilla, comprise the major proliferating tissue of the wool fibre-follicle complex. Blood vessels supply the dermal papilla and the connective sheath lining the follicle (Ryder, 1955, Nay, 1966). However, it may be that only large follicles may have their papillae invaded by blood vessels (Hynd, 1994, pers com).

Wool production per unit area is related to fibre volume (Scobie and Woods, 1992). Fibre volume in turn is a function of follicle size (Orwin, 1989), the number of cells in the bulb, cell size, and the amount of intracellular protein (Hynd 1989). Differences in cortical cell numbers appear to contribute more to the variations in fibre cross sectional area than do differences in cortical cell size (Schinckel, 1961). The number of cells going into fibre production is dependent on the number of cells committed to the fibre (cellular efficiency) and the rate of cell production (mitotic rate, Black and Reis, 1979, Wilson and Short, 1979, Hynd, 1989). After cell division, cells can also be lost by perishing or resorption (Orwin and Woods, 1982).

Variation in the partitioning of follicle bulb cells to fibre or inner root sheath has been found between animals (cellular efficiency, D.R. Scobie and P.I. Hynd, unpublished). The mitotic activity of wool follicles also varies widely between sheep and responds to nutrition (Hynd, 1989). However, it is generally accepted that cellular efficiency is genetically predetermined and not affected by nutrition (Wilson and Short, 1979, Hynd, 1989). Cellular efficiency and the mitotic rate of bulb cells normally account for 96% of the differences in fibre volume (a parameter highly correlated with fibre diameter).

Interestingly, Williams and Winston (1987) reported an interaction between the genetic capacity of wool growth and nutrition, on cortical cell volume. High wool producers increased cell volume by 24% (in response to improved nutrition) while low wool producers showed virtually no change. The rate of incorporation of cells into the cortex was also greater (20%) in the high wool producers.

2.5 Cell types of the wool fibre and fibre tenacity

Fibres often break at their narrowest point (Anderson and Cox, 1950, Fitzgerald *et al.*, 1984, Orwin *et al.*, 1980, 1985). However, fibres of similar cross sectional area can still differ in tensile strength. Since the cortex provides the bulk of the fibre, its cellular organisation and composition are likely to make a significant contribution to fibre tenacity. Importantly, Orwin *et al.* (1985) found that when a fibre breaks, rupture generally occurs within cortical cells rather than along cellular membranes. This suggests that composition

of cortical cells can potentially have a large effect on wool strength.

Since wool growth explains most of the variation observed in staple strength (Hawker, 1984), it has been suggested that the intrinsic material strength of wool (tenacity) represent a small component of staple strength variability. However, conclusions such as this have been contradicted by the work of Gourdie (1989). It was shown that staple strength variability in sheep equivalently maintained, was the result of variation in tenacity, not fibre cross sectional area (which is a function of fibre diameter).

There are a variety of cell types within the cortex. Little is known however, about their function, metabolic regulation, importance to wool growth or contribution to fibre tenacity. The cortex of the wool fibre contains a combination of meso-, ortho-, and para- cortical cells. These cell types are identified according to size, the distribution of the keratin macrofibrils within the cell (e.g. parallel arrangements in ortho-cortex, and whorl like patterns in para-cortex), and the uptake of methylene blue once the cell is oxidized (Orwin *et al.*, 1984). The meso-cortical cell is thought to be an intermediate cell type between the ortho- and para- types. The three cell types are arranged in cords or groups which run parallel along the axis of the fibre and their relative distribution can affect the appearance of the fibre. Chapman (1965) found that doggy (straight) fibres were stronger and had more para-cortex than well crimped wools. Orwin *et al.*, (1984) also reported that fibre diameter changes and crimp are associated with the occurrence of ortho-cortical cells.

There is evidence that cellular arrangement may be related to fibre diameter and to fibre tenacity in Romney sheep (Orwin *et al.*, 1980) although there is no evidence for such a relationship in Merino sheep (Hansford and Kennedy, 1990). Cross-sectional area of ortho-cortical cells, the dominant cell type, is significantly larger than that of the meso- and para- cortical cells (Orwin *et al.*, 1984). Orwin *et al.* (1980), found that weaker fibres have more ortho-cortex at the point of break, with simultaneous decreases in para-cortex. They further equated low staple strength with a log-linear relationship between ortho-cortex and fibre diameter, while high staple strength was equated with a linear relationship between these two parameters (Orwin *et al.*, 1984). This would suggest that low strength fibres have a higher proportion of ortho-cortex cells at a specific diameter.

2.6 Proteins of the wool fibre - the keratins

Keratins are fibrous proteins which provide protective outer coverings (fur, wool,

epidermis, hair and feathers) and external appendages (claws, horns and nails). They are essentially chemically inert, resistant to deformation and resistant to severe environmental conditions. Despite the diversity of keratins (reviewed Gillespie and Frenkel, 1974), those structure which have been studied such as wool and hair, all have a biphasic arrangement. This arrangement consists of bundles of filaments (intermediary filaments, IFs) embedded in an amorphous matrix (intermediary filament associated proteins IFAPs, Fraser *et al.*, 1972).

Keratinous tissues comprise three main classes of proteins: the low sulphur proteins (IF proteins) make up the filaments, the high sulphur proteins (HS) occur in the matrix and are high in cysteine, and the third class, which is also found in the matrix, is rich in tyrosine and glycine (high glycine tyrosine proteins, HGT). Differences in the relative quantities of these three groups of proteins are for a large part responsible for producing the great variety of keratogenous tissues observed (Gillespie and Frenkel, 1974).

Wool keratins are synthesised in the keratogenous zone of developing fibres (figure 2.2). Type I IF and Type II IF chains (see section 2.6.1) form two-stranded heterodimers (Fraser *et al.*, 1972, Steinert *et al.*, 1989). Pairs of these heterodimers then join to form four stranded units. The exact arrangement of these four-stranded units within the fibril is not clear, but they are embedded into a matrix of IFAPs to form **micro**fibrils. Sets of these microfibrils are collected into **macro**fibrils which are arranged in parallel or whorl-like bundles depending on the cell type (see section 2.5).

Orwin *et al.* (1985) found that the main component of a fibre which ruptured under an extensional load was the keratin protein itself. It is likely that of the keratins, the intermediate filaments provide the main component of strength (Feughelman, 1982), since no mechanical differences were evident in wools of high and low sulphur contents (Feughelman and Reis, 1967) which differ in the relative amounts of HS proteins (Gillespie *et al.*, 1964). The exact role of HGT protein on imparting strength to wool is also unclear. Some treatments which produce weak wool do reduce the quantity of HGT proteins (Frenkel *et al.*, 1974, 1975). Yet, Gillespie and Darskus (1971) showed that wool almost devoid of HGT proteins can have normal strength.

2.6.1 Intermediate filaments (IFs) - the hard keratins

There are two types of hard keratins found in wool: type I (acidic keratins) and type II (neutral-basic keratins). Marshall and Gillespie (1989) examined more than 100 different

wool samples by 1D and 2D protein electrophoresis and failed to find any IF protein polymorphism. This suggests that the hard keratins or intermediate filament proteins are consistent in their primary structure. Compared to the matrix proteins, IF proteins are lower in sulphur and richer in lysine, aspartic acid, glutamic acid, leucine and methionine (Gillespie and Frenkel, 1974).

2.6.2 Matrix proteins (IFAPs) - soft keratins

IFAPs have been particularly interesting to those studying the biology of wool growth because their composition varies in response to nutrition (Gillespie and Reis, 1966) and between animals (Woods and Orwin, 1987). Most of the matrix proteins or intermediate filament associated proteins (IFAPs) fall into two groups: The high glycine/tyrosine proteins (HGT) and the high sulphur proteins (HS). The latter group includes an ultra-high-sulphur protein (UHS) subgroup.

The variable composition of keratin, means that the half-cystine content can range from 8-19% and the tyrosine content from <3-5% (Marshall and Gillespie, 1989). Differences in the keratin composition of Romney wool seem to stem predominantly from variations in the HS fraction (Woods and Orwin, 1987). Such differences are presumed to be for a large part genetic in origin since the bands produced by protein electrophoresis remain constant for wool taken from different body parts or produced under different nutritional conditions (Woods and Orwin, 1987).

2.6.2.1 High glycine tyrosine proteins

HGT proteins are divided into two classes (Gillespie and Darskus 1971) depending on cysteine content:

Type I cysteine poor
Type II cysteine rich.

The amount of HGT protein in a fibre is partly determined by genotype since differences have been found between breeds, and between individuals. Nutrition also affects the synthesis of HGT protein. For example, infusion of zein (a protein lacking lysine and tryptophan found in maize (corn)), depresses the synthesis of HGT proteins. As a consequence of genotypic and nutritional variation in HGT protein, the tyrosine content

can vary from 2.3 to 4.4% of the total amino acid residues (Frenkel *et al*, 1974). No treatment has been shown to increase the synthesis of high tyrosine proteins (Frenkel *et al.*, 1975).

Hewish and French (1986) demonstrated that HGT proteins were preferentially located in the ortho-cortex, particularly in those cells well above the follicle bulb. Since their expression is limited to the keratogenous zone, it is perhaps not surprising that transcriptional or translational control of HGT genes has been reported. For example, Southern Blot analysis has shown class I HGT protein genes in humans, mice and sheep. Despite the presence of these genes however, expression of one of these proteins was not detected in humans and in the Merino felting lustre strain (Rogers *et al.*, 1989).

2.6.2.2 High and ultra-high sulphur proteins

The HS family is defined as having at least a 20 mole % of cysteine, and a tendency to contain high percentages of proline residues. A subgroup within this family, the ultra-high sulphur (UHS) proteins, is defined as having 30-35 mole % of cysteine. Presence of HS protein, and particularly the presence of the UHS protein subgroup, is induced or increased by high intakes of dietary sulphur amino acids (Gillespie *et al.*, 1964, 1969). Consequently, the sulphur content of wool can vary from 2.7-4.2%. It has not been possible to produce wool of less than 2.7% sulphur (Broad *et al.*, 1970). This suggests that a certain amount of sulphur is required in the basic structure of wool. Not surprisingly, there is a highly significant relationship between the sulphur content of wool and HS proteins (Gillespie *et al.*, 1964, 1969, Reis, 1967).

Infusion of casein, methionine and cysteine into the abomasum increases wool growth and the proportion of high sulphur proteins in wool. The sulphur content of the high-sulphur proteins increased and simultaneous reductions in the amount of low sulphur protein were found in an infusion experiment of this nature (Gillespie *et al.*, 1964, Reis, 1967). Gillespie *et al.* (1969) further demonstrated that the infusion of sulphur amino acids induced the synthesis of "new" (UHS) types of high sulphur proteins.

Suggestions have been made that the synthesis of UHS protein is under separate metabolic control from the synthesis of other wool proteins. This is because high methionine levels, which are inhibitory to wool growth (Reis, 1967), still allowed maximum levels of UHS protein to be reached. Marshall and Gillespie (1989) speculated on the nature of this separate control mechanism on the assumption that in the keratogenous zone, these

proteins are synthesised last. They suggest that cysteine and methionine diffuse down a concentration gradient before they are incorporated into wool protein. After supplementation with sulphur amino acids or alternatively, during periods of low wool growth, relatively more sulphur amino acids reach the site where they are incorporated into UHS protein. The relative contribution of high sulphur proteins to wool is therefore increased. Supporting this theory, is evidence from fleece weight selection lines which shows that negative correlations exist between sulphur level and clean fleece weight (Piper and Dolling, 1966, Reis *et al.*, 1967). Since the total sulphur output was greater this suggests that as wool growth increases, sulphur amino acids become limiting thereby producing a relative decrease in the % of HS proteins.

Tender wools have a greater proportion of ortho-cortex at a given diameter and contain less cysteine (Orwin *et al.*, 1980). This would suggest that para-cortical cells are richer in HS proteins than ortho-cortical cells. Orwin *et al.* (1984) and Hynd (1989), suggested that the level of nutrition regulates differentiation of cells into either of the two types by regulating the types of wool protein produced. In support, a comparison of sheep on low and high sulphur amino acid diets, revealed that higher levels of sulphur amino acids resulted in larger fibre diameters and less ortho-cortex (Hynd 1989). Hynd (1989) proposed that once ortho-cortical cells have reached their genetically predetermined maximum size, good nutrition results in a change in cell type: namely, an increase in the higher sulphur amino acid containing para-cortical cell. Since ortho-cortex contains more HGT protein compared to para-cortex (Hewish and French, 1986), the inverse relationship observed between the levels of UHS and HGT proteins, (e.g. Reis, 1967), supports an ortho/para switch.

2.7 Metabolism in the wool follicle

Wool follicles are embedded in skin which is a highly metabolic tissue. Despite it accounting for only about 10% of whole body weight (Harris *et al.*, 1989), it contributes 20% of whole body protein synthesis in lambs (Davis *et al.*, 1981) and 10-20% in mature sheep (Harris *et al.*, 1989). Although wool follicles are a separate tissue from skin, it seems likely that metabolism of the two are closely related.

Features which make the skin and wool follicle unique from other tissues include limited blood flow and O_2 supply. To illustrate, only 7-8% of cardiac output flows to the skin (Hales 1983, Harris *et al.*, 1988), and skin only uses 1-2% of whole body O_2 consumption

(Harris *et al.*, 1988). Vasoconstriction of vessels near the skin is an important means of thermoregulation. However, this reduces oxygen and nutrient supply. Consequently, although anaerobic catabolism is highly inefficient, the ability of skin to metabolise significant amounts of substrate anaerobically (Leng and Stephenson, 1965, Harris *et al.*, 1989) may be a useful adaptation to variability in its blood supply. Wool follicles have been shown to oxidize glucose and acetate *in vitro* (Leng and Stephenson, 1965) and *in vivo* (Harris *et al.*, 1989), both of which are readily available from the blood. While significant amounts of lactate are produced, pathways of glycolysis also occur (Leng and Stephenson, 1965, Harris *et al.*, 1989). Similarly, the ability of the stratum corneum of the skin (Rothman, 1954) and follicle (Brown-Falco, 1958, Philpott and Kealey, 1991) to store glycogen may also be an adaptation to prevent the shortage of energy supply.

2.8 Chemical defleecing: manipulating wool strength

Wool strength can clearly be shown to be a function of wool growth by experiments aimed at developing chemical defleecing agents. Such agents include hormones (e.g. cortisol and EGF) and methionine analogue which have the potential to result in a cessation of wool growth which precipitates a break in the fleece, most probably via inhibition of mitotic activity (Ward and Harris, 1976). The wool can be harvested by hand or mechanical means and has the main advantage of reducing animal suffering through the prevention of lacerations. To the 'shearer' the work is also less strenuous. Work is currently evaluating and refining the use of EGF to biologically harvest wool (reviewed Young and Field, 1988, Panaretto *et al.*, 1989). Some of the experiments exploring this area follow (for EGF research see section 2.3.8).

Infusion of the amino acid analogue mimosine for 1.5-2 days caused a 5-13 day cessation in wool growth (Reis, 1975). The resultant break allowed harvesting of the wool within two weeks. Unfortunately, *in vivo*, the doses required to defleece produced adverse side effects, including death (Reis *et al.*, 1973, 1975). The primary action of mimosine in vivo appears to be via inhibition of follicle DNA synthesis and cell division as determined by [^{3}H] thymidine incorporation into DNA by skin slices (Ward and Harris, 1976). Reis and Chapman (1974) further investigated chemical defleecing by using the anti-tumour drug cyclophosphamide. A single dose was required to defleece the animals by hand after eight days. Several methionine analogues also reduce wool growth. For example, ethionine causes shedding of the fleece which may be mediated by a conversion of ethionine to S-adenosyl ethionine (Reis *et al.*, 1983). Again, there is a narrow margin

between administrating toxic and non toxic effective doses. Because of this, ethionine was also found to be an unacceptable method of defleecing (Reis and Tunks, 1982).

Apart from an acute decrease in mitotic rate, a lack of high-tyrosine proteins in the wool has been implicated in reducing wool strength as the result of defleecing agents. Infusion of zein into the abomasum, a treatment known to produce weak wool (Frenkel *et al.*, 1975), suppresses the synthesis of high-tyrosine proteins (Frenkel *et al.*, 1974). In addition, mice which are heterozygous for a naked gene mutation, produce weak hair which is shed continuously and is low in high tyrosine proteins (Tenenhouse, 1974). Gillespie and Darskus (1971) shown however that wool almost without high tyrosine protein can have normal strength. Furthermore, Reis and Gillespie (1985) found that administration of ethionine produced weak fibres but the proportion of HGT protein in these fibres was not affected.

Experimental design, animal selection, and animal care

3.1 Research overview

Research for this thesis consisted of three major experiments. Following is a brief description of each trial together with its principal aim (see also Fig 3.1). Trial 1 evaluated endocrine and metabolic parameters as markers of staple strength (chapter 5). These parameters included several indicators of insulin status because previous work with animals of similar genetic origin, had demonstrated a correlation between staple strength and plasma insulin (Armstrong 1989, unpublished). The experimental design of Trial 1 allowed potential markers to be evaluated between genders and within two experimental environments (indoor versus outdoor). This was so that these environments could be evaluated for future use. Environments differed with respect to feeding regimes (once daily feeding of pellets versus pasture *ad lib*) and exposure to the elements (outdoor animals were exposed to wind and rain).

The results from Trial 1 indicated that plasma insulin was a marker of wool strength. Consequently, the second experiment (Trial 2) concentrated on insulin action at the tissue level by measuring insulin receptor binding (chapter 6). Trial 3, used the same animals as Trial 2 and was designed to study gene expression in the wool follicle of some loci potentially involved in glucose uptake (chapter 7). Such expression was compared between high and low wool strength animals before and after an insulin infusion.

3.2 Breeding trial

Heritability figures for staple strength from Romneys are comparatively high and have been estimated at 0.52 (Hawker *et al.*, 1988) and 0.58 (Bigham *et al.*, 1983). As a comparison, heritability figures for other production traits such as greasy fleece weight, mean fibre diameter, hogget live weight and staple length are estimated at 0.44, 0.33, 0.47 and 0.34 respectively (Hawker *et al.*, 1988). The high estimates of staple strength

heritability have led to the development of a staple strength breeding program. This program was initiated by AgResearch and WRONZ during 1986 at the AgResearch Templeton Research Station. The aim of the program was to develop high and low staple strength lines (Orwin and Geenty, 1986, Rogers *et al.*, 1990). Significant divergence in SS between the lines has been observed and Rogers *et al.*, (1990), have shown a divergence in length after carding between high and low staple strength lines after only two seasons of selection. A more recent study (Bray *et al.* (1992)) reported that the high line showed a 25% increase in staple strength over the control line while the low line showed a 15% reduction. Animals for this thesis were selected from both lines.

There were several advantages in using the breeding programme to supply animals. Sibling, dam and sire staple strength data, sometimes over several years, were available to calculate breeding values for staple strength. This allowed animals to be selected based on their genetic potential for staple strength. Lamb and hogget data were also available before selection thereby allowing phenotype to be assessed. Because the breeding programme included both high and low wool strength lines, a wide range of staple strengths was encountered. This allowed the selection of animals with extremely low and high staple strength which were more likely to show differences in markers of wool strength. Lastly, facilities and some techniques to be used in the experiments had previously been established at the site of the breeding program.

3.3 Parameters used in animal selection

3.3.1 Staple strength

Several parameters are used in the industry as an indicator of wool strength: in this work the parameter used was staple strength. Staple strength is defined as the tensile strength of a length of fibre requiring spinning and twisting to form a yarn. Other useful indicators of wool strength are length after carding (LAC, mechanical untangling and straightening of fibres before spinning) and individual fibre strength. LAC is the direct consequence of strong or weak wool in the industry and it is during this process that weak wools cause the most problems (Orwin and Geenty, 1986). However, LAC is time consuming to measure and requires at least 800 g of a full-length fleece. Similarly, individual fibre strength is also a more direct measure of processing performance, but is equally time consuming to obtain.

Staple strength measurements are affected by the stress strain curve of the fibres, the configuration of fibres within the staple (i.e. crimping), the gauge length of the apparatus (the length of the staple between the clamps which break it), broken fibres and cotting. Nevertheless, it has been shown that staple strength is an important component in predicting length after carding (Gee, 1978, Orwin *et al.*, 1987). The method is also fast, requires small amounts of wool, and the wool need not represent a full year's growth. Staple strength is expressed as the peak load (Newtons) to break a staple of a given linear density (ktex). Traditionally, wools below 25 N/ktex are referred to as tender (weak) and those above 30 N/ktex as sound (strong).

3.3.2 Measurement of staple strength

Fleeces were harvested in the first week of November each year. To measure staple strength, five sets of neighbouring greasy staples or portions of the same staple (depending on staple size) were selected from a mid side sample. The mid-side was selected as a 10 cm^2 area with a centre on the last rib approximately 18 cm down from the backbone. Greasy staple linear density (ktex/B) was measured at the visually predetermined region of break using a pneumatic fibre thickness gauge. Only staples in the 1.77-1.88 ktex range were broken. When extending the staples, care was taken not to pre-stress the staple by ensuring some slack was present in the staple before clamping. Staples were broken at a constant extension rate (100 mm min^{-1}) and expressed as maximum load to break (N/ktex), one kilotex being the density of a standard yarn weighing 1g/metre). The apparatus consisted of an electrically powered modified Agritest tensile tester (Agritest Pty Ltd Australia), with a constant gauge length of 40 mm. Tests were carried out at 65% relative humidity and 20 ^0C after the preconditioning of samples to this environment for 24 hours.

3.3.3 Breeding value for staple strength

Breeding values are a useful statistic for assessing the genetic worth of an animal for a particular trait. It indicates the individual's ability to pass on the trait of interest to its progeny. Breeding values are calculated using information which is sensitive to environmental factors. However, the averaging effect obtained from the inclusion of figures from many relatives over several years gives a closer measure of genetic rather than environmental effects. In a population, the average breeding value is zero. Animals

with negative staple strength breeding values (SSBVs) have below average breeding performance and those with positive SSBV's have above average breeding performance. The breeding value used in this work considered staple strength information from an animal's relatives as well as its own hogget staple strength.

3.3.4 Calculation of staple strength breeding value

Breeding values were calculated by Dr Neil Clarke of AgResearch, Ruakura using a best linear unbiased prediction (BLUP, Henderson *et al.*, 1959, Garrick, 1991). This parameter gives an estimate based on: the staple strength of an individual animal (calculated from the hogget fleece), and the staple strength of its paternal half-siblings (calculated from the hogget fleece).

3.4 Trial structure (see figure 3.1)

3.4.1 Animal selection

3.4.1.1 Trial 1 (1990)

Animals were selected using staple strength information from the 1989 wool clip. Variation in wool strength was exploited to provide the most useful data for the correlative analysis used. Consequently, maximum variability among animals was the criterion used to select the Trial 1 rams. Staple strengths for this group ranged from 13.59 - 79.10 N/ktex for the outdoor group and 6.34 - 52.79 N/ktex for the indoor group. The ewes, ranging in staple strengths from 16.37 - 81.23 N/ktex, were selected as 1987 born half-siblings to the outdoor rams. Eighty percent of the ewes lambed, the remainder were dry.

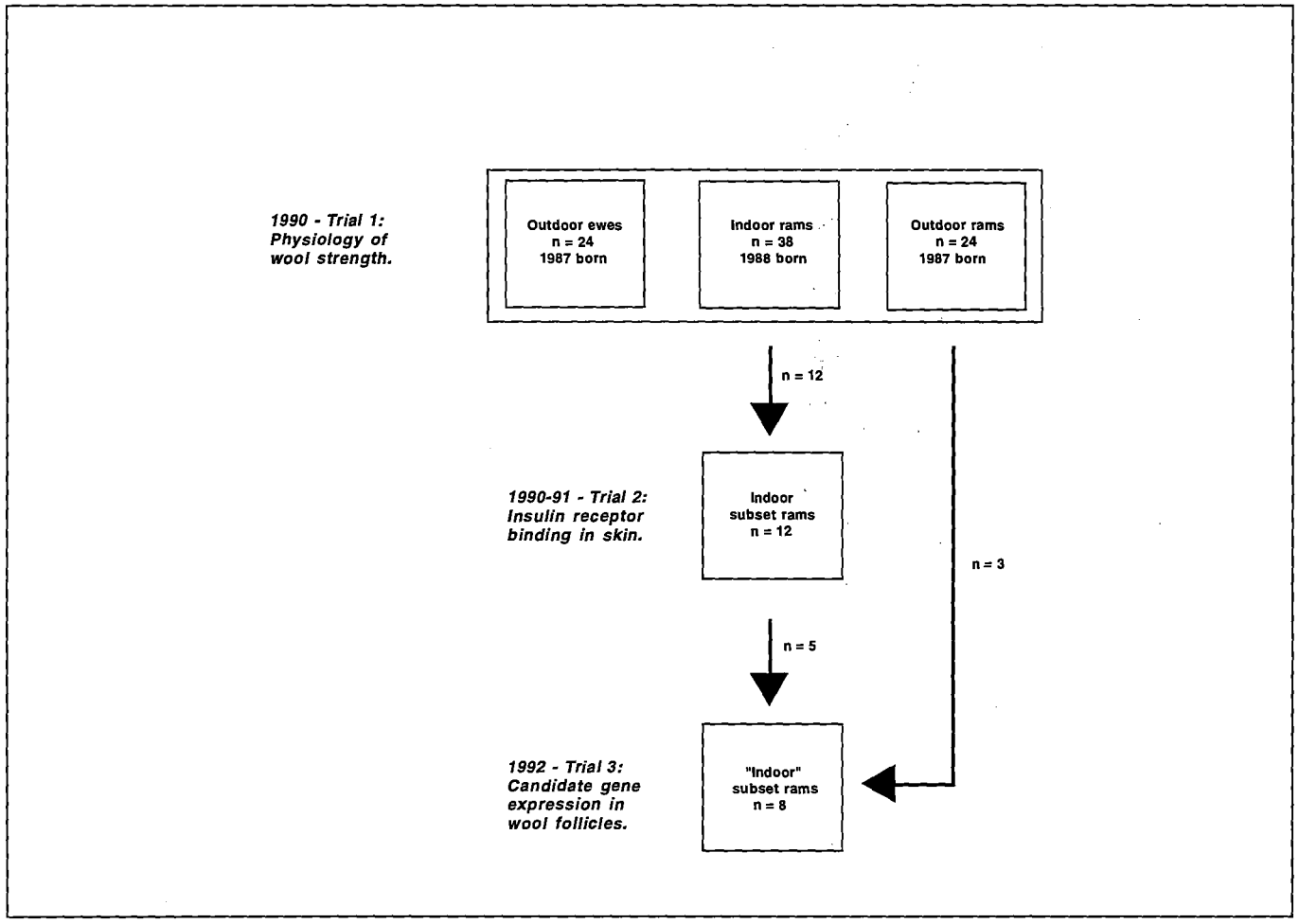


Figure 3.1: Overview of the animals used in each of the experimental trials.

3.4.1.2 Trial 2 and 3 (1991-2)

Trial 2 consisted of 12 animals selected to have a range in breeding value for staple strength (-10.22 - +14.25 N/ktex). They were chosen from the 38 rams which were housed indoors during Trial 1. These animals are called the subset rams throughout this thesis. Animals were selected to provide extremes in breeding value thereby likely to have genes for extremely high and low strength wool. Two deaths occurred in Trial 2 during 1991 because of unexplained difficulties in the administration of general anaesthesia. As the result of this, three further rams were selected for Trial 3 from the 1990 "outdoor" rams.

3.4.2 Animal management

3.4.2.1 Indoor

3.4.2.1.1 Trial 1 and 2 (AgResearch, Templeton)

Housing was provided in adjacent naturally lit individual pens which allowed visual, auditory and some physical contact between animals. Pellets were fed at the rate of 53-60 $g/kg^{0.75}$ at 8.00 a.m. Water was available *ad libitum*. Pellets consisted of 40% lucerne hay, 27% barley straw, 10% oats, 10% peas, 10% barley, 1% lime, 1% salt and 1% molasses. Nutritional value of the pellets was assessed as 87.7% dry matter, 7% ash, 12% crude protein, 30% detergent fibre and 52% neutral detergent fibre. To allow nutritional equilibration and familiarization with feeding and handling procedures, animals were acclimatized to their environment for three weeks before commencement of sampling. Live weights for the calculation of daily allowances were redetermined weekly.

3.4.2.1.2 Trial 3 (AgResearch, Palmerston North)

Animals were released onto pasture following transportation to Palmerston North from Templeton research station. A commercial, fully enclosed carrier provided the transport. Approximately five days before they were due for surgery, each animal was collected and housed indoors in metabolic crates. While inside, rams were fed 900 grams of lucerne chaff daily except on their day of surgery. Rations were mostly consumed by 12.00 noon Water was available *ad libitum*.

3.4.2.2 Outdoor

3.4.2.2.1 Trial 1

Pregnant ewes and their half-sib rams were grazed together in one mob on a rye-grass clover pasture. They were rotated to different paddocks to maintain the average live weight for the group. Live weights were measured weekly.

3.4.2.2.2 Trial 2 and Trial 3

Trial 2 rams were grazed in one mob between indoor trials. They were not used for mating, nor were they used for experiments other than those described in this thesis.

Relationships between wool production traits and the repeatability of wool strength between years

4.1 Introduction

Wool is an extremely variable commodity. To determine the suitability of a fleece for a specific application, it is assessed for traits such as yield and fibre diameter. Other common industrial indicators of quality (or lack of) are fleece yellowing, staple length, cotting (broken fibres), staple strength and bulk (Hansford, 1992).

Desirable traits have resulted in the initiation of many selection trials. Unfortunately, selection for one beneficial parameter may reduce the occurrence of another. For example, Moore *et al.* (1989), found that selection for fibre diameter with the aim of increasing fleece weight resulted in the follicle density, another desirable trait, being halved.

Work described in this chapter aimed to establish if undesirable changes in wool characteristics occur under selection pressure for staple strength. Consequently, greasy fleece weight was measured because it correlates highly to clean fleece weight and yield, and live weight was measured since it is a correlant of carcass weight (and greasy fleece weight via skin surface area). Furthermore, minimum fibre diameter (for wool grown in July) was included since it predicts processing performance and length after carding. It has been reported that in Merino sheep rate of fibre diameter change explains slightly more variability in staple strength than minimum fibre diameter alone (Hansford and Kennedy, 1988). However, although rate of fibre diameter change would perhaps have been a more accurate predictor of staple strength, its measurement (Hansford *et al.*, 1985) was beyond the scope of this thesis.

A second objective of this chapter was to assess the repeatability of wool strength. Variation in staple strength between individuals results from an interaction between genotype and environment which produces the phenotype. To get an idea of the relative importance of genetic components in producing the phenotype, the consistency of the phenotype as the environment changes was determined by observing the consistency of staple strength across years.

4.2 Methods

4.2.1 Animal care and selection

Animal selection and care for these experiments are described in chapter 3 (Trials 1 and 2, sections 3.3 and 3.4).

4.2.2 Staple strength and its breeding value

The staple strength measurement and calculation of its breeding value are described in chapter 3, sections 3.3.2 and 3.3.4 respectively.

4.2.3 Minimum fibre diameter

Samples were measured by Whatawhata fibre testing centre using a CSIRO fibre fineness distribution analyzer (FFDA, Lynch and Michie, 1976).

4.2.4 Greasy fleece weight

Fleeces were collected after shearing and weighed.

4.2.5 Live weight

Animals were weighed by guiding the animal into a cage mounted onto a scale. No adjustment for the increases in fleece weight during the trial was made.

4.2.6 Statistical analysis of fleece data

Pearson product-moment correlation coefficients (R) between the various parameters were calculated using the SAS correlation procedure (SAS, version 6.07, SAS Institute Inc, SAS Circle, Cary, NC 27512-8000 USA).

4.3 Results

4.3.1 Relationships between parameters of wool quality

An increase in minimum fibre diameter, resulted in an increase in staple strength. This relationship was seen for both indoor and outdoor rams (Tables 4.1 and 4.2, Figure 4.1) and outdoor ewes (Table 4.1). Correlation coefficients for this association were highly significant ($p < 0.0001$) and varied from 0.67 to 0.73 so that approximately 49% (R^2) of the variation in staple strength could be explained in terms of variation in fibre diameter. A similar correlation ($R = 0.65$, $p < 0.01$) was seen between mean staple strength and mean minimum fibre diameter (1989-91 Figure 4.1) for the subset rams.

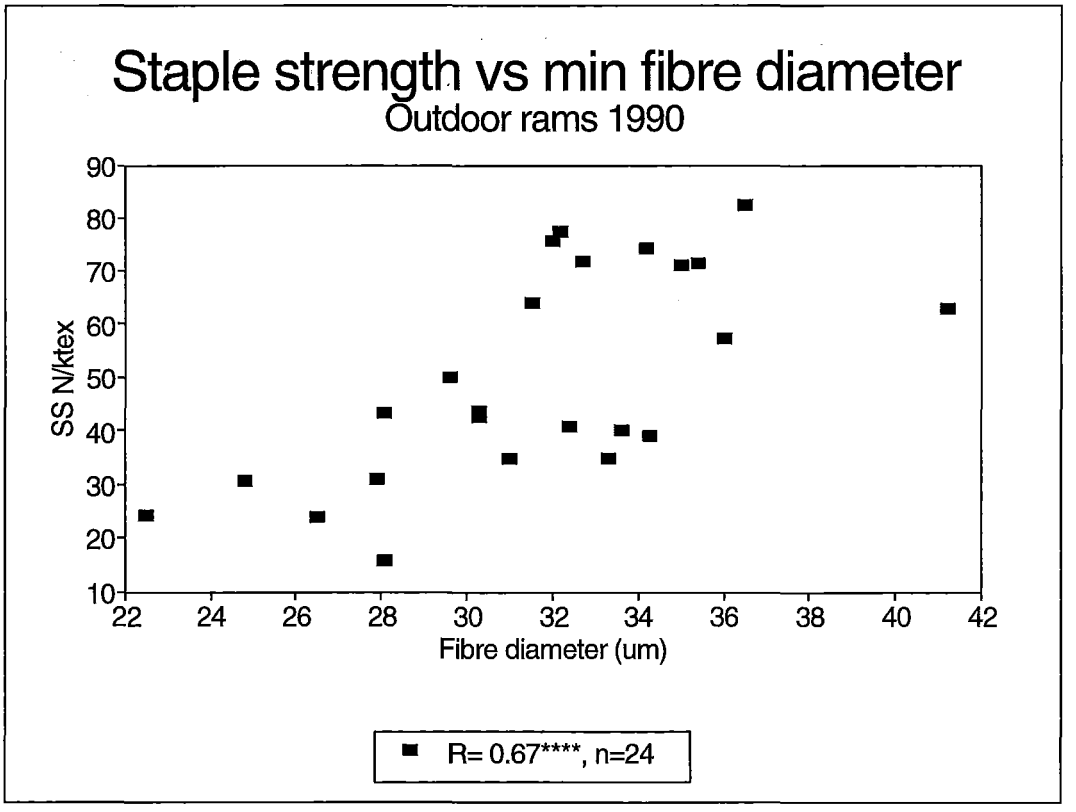


Figure 4.1: Scatterplot of staple strength graphed against fibre diameter.

The fibre diameter value was derived from wool grown during July and will therefore represent wool within the winter break region. The correlation coefficient shown (R) is significant ($p < 0.0001$).

	SSBV		SS		minFD		GFWT	
	♂	♀	♂	♀	♂	♀	♂	♀
LWT	0.26	-0.02	0.37*	0.50**	0.45**	0.52***	0.44**	0.68****
GFWT	0.17	0.00	0.38*	0.55***	0.38*	0.39*		
minFD	0.60***	0.60***	0.67****	0.73****				
SS	0.74****	0.33						

Table 4.1: Association between parameters of fleece quality for the outdoor rams and ewes, 1990

Pearson correlation coefficients are shown (R) as well as the level of significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$), n = 24 for both rams and ewes.

Low, but significant correlations ($0.001 < p < 0.05$) between greasy fleece weight, and staple strength or minimum fibre diameter were found in the outdoor rams and ewes (tables 4.2). These results could not be presented for the indoor group since parts of the fleece were removed for sampling during the year.

	SSBV	SS	minFD
LWT	0.07	-0.09	-0.12
minFD	0.38*	0.67****	
SS	0.51****		

Table 4.2: Association between parameters of fleece quality for the indoor rams, 1990

Pearson correlation coefficients (R) are shown as well as their level of significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$), $n = 38$.

Treatment affected the relationship between fleece traits and live weight. It was found that live weight correlated to staple strength and minimum fibre diameter in the outdoor, but not the indoor animals (Tables, 4.1 and 4.2).

4.3.2 Repeatability of wool strength between years

Mean staple strength for the subset rams (Table 4.3) varied considerably between years. Whereas fibre diameter increased every year, staple strength increased from 1989 to 1990 and decreased from 1990 to 1991.

Comparison of staple strength between 1989-1990 and 1990-1991 showed that an individual's staple strength was moderately consistent across years ($R = 0.57$ and 0.63, $n = 12$, $p < 0.01$). Similar comparisons for fibre diameter revealed that annual minimum fibre diameter measurements for an individual across years were highly consistent ($R = 0.80$, $p < 0.001$ and 0.94, $p < 0.0001$, $n = 12$).

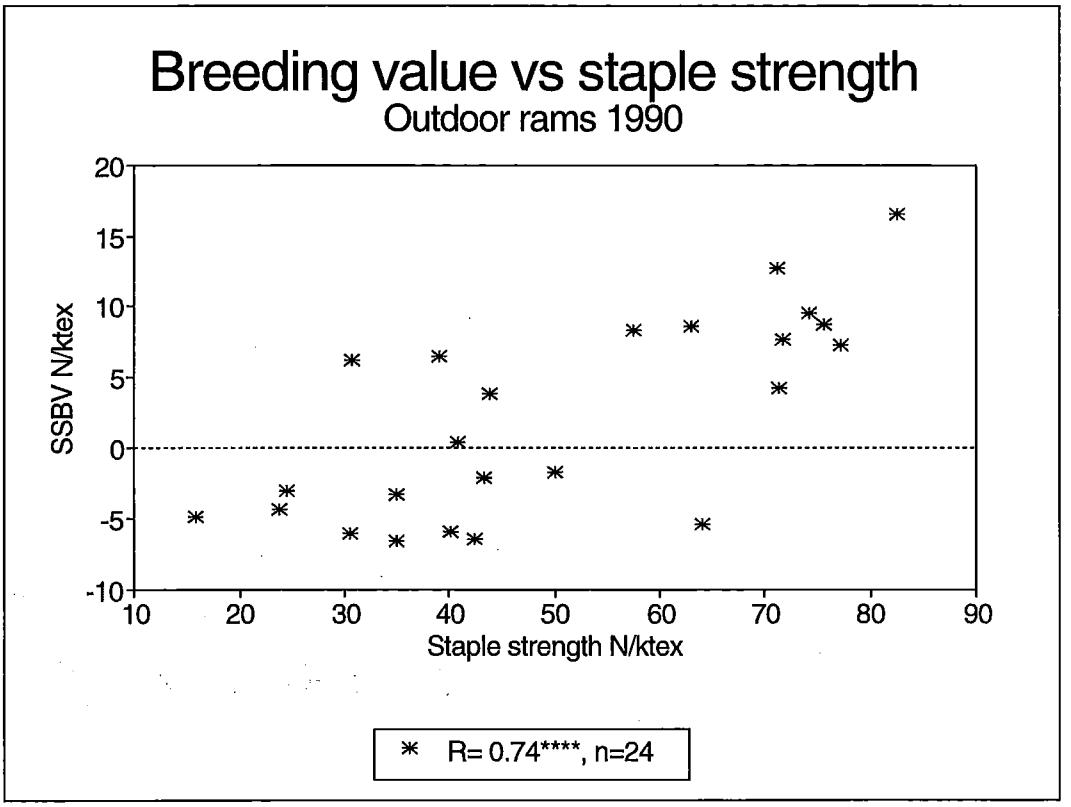


Figure 4.2: Scatterplot of staple strength breeding value graphed against staple strength.

The dotted horizontal line represents a breeding value of 0, above which animals are likely to produce progeny of higher than average staple strength, and below which animals are likely to produce progeny of lower than average staple strength. The correlation coefficient shown (R) is significant at the $p < 0.0001$ level.

In the subset rams breeding value for staple strength was highly correlated to the individual's average staple strength for 1989-91 ($R = 0.80$, $p < 0.0001$). Correlation coefficients for individual years were 0.85 ($p < 0.0001$, 1989), 0.71 ($p < 0.0001$, 1990) and 0.27 (ns, 1991), suggesting that the ability of breeding value to predict staple strength diminished with age. Additional data from Trial 1 produced the following correlations between breeding value and staple strength: $R = 0.74$ ($p < 0.0001$, Figure 4.2) for the outdoor rams, 0.33 (ns) for the outdoor ewes, and 0.51 ($p < 0.0001$) for the indoor rams (Tables 4.1 and 4.2).

Tag	1989		1990		1991		average 89-90		SSBV
	SS	minFD	SS	minFD	SS	minFD	SS	minFD	
440	10.5	26.5	52.8	31.0	49.6	34.3	37.6	30.6	-10.22
439	13.8	27.0	45.5	31.3	39.3	35.1	32.9	31.1	-9.39
448	9.6	28.1	67.0	31.4	42.8	34.6	39.8	31.4	-5.64
209	28.6	28.3	47.7	30.2	Died		38.2	29.3	-3.76
618	17.3	29.6	93.1	32.4	64.1	34.9	58.2	32.3	-3.34
621	27.2	30.1	88.9	35.6	61.9	40.5	59.3	35.4	-1.95
640	31.8	29.5	71.6	32.8	36.8	38.3	46.73	33.5	0.02
623	27.7	28.3	65.2	30.9	55.4	34.0	49.4	31.1	4.56
617	21.7	26.9	74.4	28.2	38.1	29.1	44.73	28.1	5.56
604	28.7	28.8	92.3	33.4	Died		60.5	31.1	6.22
415	52.1	26.5	90.4	31.6	48.3	35.1	63.6	31.1	9.13
641	52.7	31.1	98.7	36.5	63.5	39.6	71.63	35.7	14.24
mean	26.8	28.4	74.0	32.1	50.0	35.6	50.2	31.7	0.45
SEM	4.07	0.43	5.43	0.66	3.39	1.03	3.53	0.64	2.18

Table 4.3:

Staple strength (N/ktex), minimum fibre diameter (μm) and breeding value data for the subset rams 1989-91 (N/ktex)

4.4 Summary of results

- Minimum fibre diameter was an important determinant of staple strength.
- The magnitude of the relationship between staple strength and minimum fibre diameter was independent of gender and management regime.
- Where intake was fixed in a live weight dependent manner, minimum fibre diameter, or staple strength, did not correlate to live weight. These parameters did correlate in the outdoor animals.
- Where the comparison could be made (outdoors only), animals with higher liveweight had higher greasy fleece weight.
- Ewes did not show a significant relationship between staple strength and their breeding value for staple strength.
- Staple strength was affected by environment as illustrated by the variation in staple strength means across years.
- The staple strength or minimum fibre diameter ranking of an animal within the flock was moderately consistent across years.
- The correlation between staple strength and breeding value declined with age.

4.5 Discussion

4.5.1 Relationships between production traits

Results show that there is a positive association between minimum fibre diameter (winter break) and staple strength. In support, others have demonstrated that high staple strength wools have greater mean fibre diameter, as well as longer staples, and longer fibre length after carding (Anderson and Cox, 1950, Ross, 1965, Fitzgerald *et al.*, 1984, Hawker and Littlejohn, 1989, Rogers *et al.*, 1990).

In the industry a high tensile strength to fibre diameter ratio is desirable because fibre coarseness can restrict a product's versatility and value. However, the observed increase in fibre diameter with higher staple strength observed in this work is unlikely to have an effect on price. Wiggins (1986), reported that the effect on price of fibre diameter variability in the 28-36 μm range, is negligible. This is because the importance of fibre diameter in view of quality and commercial value, declines with fibre diameters of 25 μm and above (Maddever *et al* 1991). Premiums for an increase in length after carding however, are significant. Maddever *et al* (1991), indicated that the increase in price after a 1 mm increase in length after carding is 0.3 c/kg. This is based on a clean wool price of 450 c / kg. In a recent report, a staple strength difference of 11 N/ktex was accompanied by a 2.6 μm change in mean fibre diameter and 13 mm (Hauteur) to 15 mm (Barbe) difference in length after carding (Rogers *et al.*, 1990). According to Maddever *et al.* (1991) this would have resulted in an increase of 5 cents/kg clean wool.

Wool strength was mildly correlated to fleece weight and selection for staple strength should therefore result in heavier fleeces and higher financial returns (assuming that there is a genetic correlation). Other workers have reported that high fleece weight is associated with greater staple strength (Hawker *et al.*, 1988, Hawker and Littlejohn, 1989, and Bray *et al.*, 1992). Such increases in fleece weight were usually associated with increases in fibre diameter and fibre length (Hawker *et al.*, 1988, Hawker and Littlejohn. 1989). Fibre diameter was correlated to fleece weight in this study. This is not surprising given that a correlation between staple strength and fleece weight was also observed.

Live weight and staple strength were correlated in the outdoor but not the indoor animals. This could suggest that for the outdoor treatment high wool strength animals had higher intake resulting in higher live weight. However, no changes in mean live weight were

observed. This therefore hinders the interpretation of this finding. Certainly, studies by others have not shown a correlation between staple strength and an ability to lose or gain live weight in response to nutritional treatments (Hawker and Littlejohn, 1989, Bray *et al.*, 1993).

Sumner and Hawker (1986) concluded that gender effects on objective wool characteristics were of no practical significance after allowing for body size and intake. The results in this thesis confirm their findings. There were no differences in fibre diameter or staple strength between rams and ewes (Table 3.3). Rams had higher fleece weights, but this is probably due to greater skin surface area as the result of higher live weight. Thus, in future experiments, it seems valid to use rams to study staple strength as being representative of a largely female population.

4.5.2 Repeatability of wool strength

Staple strength varied from year to year. For example, staple strength in the subset rams almost tripled between 1989 and 1990 and fell in 1991. Surprisingly, these changes were not reflected in proportional changes in minimum fibre diameter. Instead, mean minimum fibre diameter for the subset rams steadily increased from 1989-91.

These results suggest that significant decreases in intrinsic material strength may have occurred in the 1991 wool clip. 1990 was the year in which the subset rams were used for an indoor trial and their nutrition and housing was different from 1989 and 1991. Gourdie (1989) and Gourdie *et al.*, (1992) however, concluded that intrinsic material strength does not change with nutritional treatments. An alternative explanation must therefore be sought. It is possible that the changes in intrinsic material strength reflect direct exposure to winter weather. During 1990, the year in which the subset rams showed the highest staple strength, animals had been kept in an indoor environment from late May until mid September thereby increasing staple strength. When these same animals were put out to pasture in the subsequent year staple strength fell. Alternative explanations for variability in staple strength which are independent of intrinsic material strength or minimum fibre diameter include variability of cross sectional area along the fibre (Collins and Chaikin, 1971), the configuration of fibres within the staple (eg crimp, Van Luijk, 1984), the stress strain curve of the fibre (reviewed Gourdie 1990), fibre-fibre interactions (cotting, Orwin *et al.* 1987) and rate of fibre diameter change (Hansford and Kennedy, 1988).

Minimum fibre diameters record correlated well between years, but similar correlations for

staple strength were not as high. Repeatability for staple strength was comparable however, if not higher than that observed by Ross (1965). The repeatability of both staple strength and minimum fibre diameter suggests that there could be a large genetic element involved in imparting staple strength. An alternative interpreation of this result would be that a permanent environmental effect had a large bearing on staple strength (e.g. weight at birth, 2^0 to 1^0 follicle ratio). Furthermore, these data suggest that staple strength is more susceptible to change by factors other than genotype than minimum fibre diameter.

SSBV correlated highly with staple strength for the outdoor rams, poorly for the outdoor ewes and moderately for the indoor rams (Tables 3.1 and 3.2). In addition correlations between breeding value for staple strength and staple strength diminished with age. A possible limitation to the breeding value is that its owner's sole contribution to the value is its hogget fleece weight. The measurement consequently does not allow for changes in gene expression associated with maturation, e.g. age and reproductive status. In support, Lewer *et al.* (1983) found that a low genetic correlation existed between hogget and lifetime average fleece weight. Given that both fleece weight and staple strength are related to wool growth, it is not unlikely that hogget staple strength is also a poor predictor of lifetime average staple strength. Repeatability of fibre diameter on the other hand, was higher between age one and two, as compared to fleece weight ($R = 1.00 \pm 0.44$, cf 0.41 ± 0.25, Lewer *et al.*, 1983), a finding supported in this work.

More work is required to determine the usefulness of a breeding value for staple strength where only a single record from the individual is included in the estimate. For the ewes future studies should calculate breeding values from data which is adjusted for various environmental effects such as total lamb weight at weaning. It may also be necessary to update breeding values every year before their full benefit is realised. Alternatively it may be useful to investigate if a breeding value for minimum fibre diameter provides a better indication of lifetime staple strength.

Biochemical markers of seasonal wool growth

5.1 Introduction

It has previously been shown that genetic differences in wool growth do not express themselves as differences in alimentary tract metabolism. For example, Piper and Dolling (1969), found no differences in the ability of high fleece weight and control lines to digest a variety of diets and instead concluded that efficiency of wool growth was different (the ability to convert nutrients to wool after the digestion process). Further evidence for post digestive changes being responsible for differences in wool growth was provided by Lush (1992) who set out to establish if absorption of metabolites from the gut explained variation in wool growth capacity. She found no differences in nutrient supply from the gut to the rest of the body in high and low wool growth lines and concluded that post digestive changes in nutrient supply to the skin (eg. wool follicle function) were responsible for the difference in wool growth between the two lines.

The works of these authors implicate the mechanisms by which energy and nutrients are partitioned to and taken up by the wool follicle as being of primary importance in the regulation of wool growth. The potential role of the endocrine system in regulating such partitioning is substantial since the endocrine system has been shown to regulate the partitioning of nutrients to other tissues (Black and Reis, 1979, Oddy and Lindsay, 1986, Godfredson *et al.*, 1990, Wynn *et al.*, 1990, McDowell and Annison, 1991, Bickerstaffe, 1993).

Since wool growth is thought to be a highly significant factor in imparting wool strength (via an effect on fibre diameter) this chapter focuses on a search for biochemical markers of wool growth.

5.1.1 Experimental design

A number of issues were important in considering the evaluation of a measurable

endocrine or biochemical factor as a suitable marker of wool growth.

These included

a) The ability of the endocrine or biochemical factor to affect wool growth.

b) The availability of an assay procedure for the endocrine or biochemical factor.

c) The cost of the assay.

Because of the unavailability of local assays, EGF and catecholamines levels were not assessed despite evidence that they can affect wool growth.

One challenge was to select a suitable sampling procedure for hormones known to have a diurnal rhythm, e.g. cortisol, insulin, melatonin and growth hormone. To this end, a single sample taken at a fixed time each week for many animals was seen as giving more useful information than sampling fewer animals more intensively, since the resourses were not available (time and money) to intensively sample the number of animals required. The assumption was therefore made that between animal temporal variation would be minimised with large animal numbers.

5.1.2 Biochemical background to screening parameters

5.1.2.1 Metabolites

5.1.2.1.1 Glucose

Glucose does not constitute the primary energy source within the blood stream of sheep. Instead, short chain fatty acids (acetate, propionate and butyrate), ketone bodies, and non esterified fatty acids are the main energy supply to ruminant tissues (Blaxter 1962, Payne and Payne 1987, McDowell and Annison 1991). Nevertheless, maintenance of glucose homeostasis is just as important in the ruminant (Pell and Bergman, 1983) as it is in man. This is because the ruminant brain does not have the ability to regulate its absolute requirement for glucose. While the brain of canine, rodent and human individuals can

utilize considerable amounts of ketone bodies, sheep brain is only able to utilize glucose and small amounts of branched-chain amino acids (Pell and Bergman, 1983).

In ruminants, most of the monosacharrides ingested, are fermented by rumen microbes before they can be absorbed from the gut (Lindsay, 1970). Glucose therefore needs to be obtained from endogenous sources. These source include the gluconeogenesis pathway and the Cori cycle (which converts lactate back to glucose in the liver).

Precursors for the gluconeogenesis pathway include propionic acid (from rumen fermentation), gluconeogenic amino acids, and glycerol (from fat oxidation). In ruminants, gluconeogenesis is largely regulated by energy and precursor intake (Brockman, 1986, Wieghart *et al.*, 1986), but it can also be regulated by physiological status such as pregnancy or lactation (Weekes, 1991). Converse to the human situation, exogenous glucose supply does not regulate gluconeogenesis in sheep (Janes *et al.* 1985). The glucose produced by endogenous processes is stored in the liver in the form of glycogen.

Glucose output by the liver (via the breakdown of stored glycogen), is a highly regulated process which is inhibited by insulin and is promoted by glucagon (reviewed McDowell, 1983). In sheep, the release of glucose from the liver is not inhibited by high levels of plasma glucose (Sasaki *et al.* 1982). Instead, the most likely mechanism by which hepatic glucose output is regulated, is by the ratio of insulin:glucagon (Sasaki *et al.*, 1982).

Other hormones may also play a role in the regulation of glucose homeostasis (virtually every endocrine organ has been shown to exert some effect reviewed Brockman, 1986). The adrenal gland is particularly effective at modulating glucose levels. However, in the fed, resting, and unstressed animal, glucocorticoids and catecholamines are not required to maintain glucose homeostasis (Reilly and Black, 1973).

5.1.2.1.2 Urea

Plasma urea levels reflect the uptake of nitrogenous compounds from the digestive tract. These nitrogenous compounds can consist of crude protein derived from rumen micro-organisms as well as ingested plant material. Plasma urea levels also fluctuate according to plasma volume which is inherently related to kidney function.

Nitrogenous compounds move across the gut wall as protein, ammonia and urea (Payne and Payne, 1987). An increase in energy intake promotes the efficient recycling of urea

into microbial protein by rumen microorganisms within the gut. This is because those molecules which are efficient sources of energy also supply the carbon skeletons required for the synthesis of amino acids used in bacterial protein.

Starvation increases the degradation of muscle protein. The released amino acids can provide carbon skeletons and energy if no other sources are available. For example, Ballard *et al.* (1976) showed that starvation increased the output of amino acids from the hind-limb of sheep. Catabolism of amino acids is reflected by an increased excretion of urea since transamination reactions occur more frequently as a consequence of catabolic activity. As a result, external provision of energy alone can reduce urinary nitrogen elimination by 40-60% (Lobley *et al.*, 1987). This is termed the amino acid sparing effect. Urea levels therefore reflects both protein turnover and protein uptake.

5.1.2.1.3 Creatinine

Phosphocreatine functions as a reserve for high-energy phosphate groups in peripheral tissues, particularly in muscle and nerve cells. Its presence allows the maintenance of ATP concentrations at constantly high levels, particularly in skeletal muscle. Phosphocreatine is the phosphorylated derivative of creatine, the latter being synthesised by the liver and kidney from arginine, lysine and methionine. In clinical studies, phosphocreatine levels are often assessed by measuring creatinine. This compound is the product of phosphocreatine degradation and is formed in proportion to muscle mass from the non-enzymatic cyclization of creatine and creatine phosphate in muscle (Bender, 1985). Phosphocreatine and creatinine together amount to 0.3-0.5% of muscle weight (Linder, 1991).

5.1.2.1.4 B - hydroxybutyrate

B-hydroxybutyrate and acetoacetate are the major constituents of ketone bodies in ruminants. Ketone bodies are formed during the B-oxidation of fats in the liver and they can be seen as an overflow mechanism for when the liver cannot oxidise all the acetoacetate and β-hydroxybutyrate formed by β-oxidation. The liver therefore redistributes these products as ketone bodies to the peripheral tissues, where they act as substrates in the Krebs cycle.

In ruminants, butyrate as well as fat provides a major precursor of β-hydroxybutyrate.

Butyrate is derived from the rumen fermentation of food stuffs and provides a direct source of energy. It is converted to β-hydroxybutyrate in the rumen epithelium (Leng and West, 1969).

In humans ketone body formation is usually associated with fasting (Linder, 1991) or *diabetes mellitus*. In ruminants however, ketone bodies are normal constituents of the blood and an important source of fuel. Up to 80% of a ruminant's energy requirement can be derived from short chain fatty acids (acetate and ketone bodies, Blaxter, 1962). It therefore appears that muscle tissue in ruminants is well adapted to using ketone bodies as an energy source.

5.1.2.2 Hormones and growth factors

Below is a brief description of the physiological role of the endocrine candidate markers selected for screening purposes. Their potential involvement in wool growth was discussed in the literature review.

5.1.2.2.1 Insulin

Insulin is produced by the Islets of Langerhans of the pancreas and is the primary hormone responsible for glucose homeostasis. In ruminants, insulin promotes the uptake of glucose and short chain fatty acids from the blood (Jarrett *et al.*, 1974), as well as promoting muscle protein deposition, possibly via stimulation of amino acid uptake (Brockman *et al.* 1975). Insulin reduces hepatic glucose output in ruminants (Brockman, 1983) which is likely to occur via modulating gluconeogenic precursor uptake (both in type and amount) as well as stimulation of hepatic glycogen synthesis (Brockman, 1986).

Insulin has a large effect on both muscle and adipose tissue metabolism in sheep. In muscle, protein deposition is achieved both by stimulating protein synthesis and inhibiting protein degradation (Prior *et al.*, 1984). Conversely, low insulin concentrations increase the output of amino acids by the hind limb (Ballard *et al.*, 1976). Acetate is a major substrate for fat synthesis. Insulin injections into normal sheep reduce plasma acetate levels (Call *et al.*, 1972) and have been found to decrease ketogenesis by reducing free fatty acid supply to the liver (Jarrett *et al.* 1974). Insulin also suppresses the production (Brockman, 1976) and utilization (Jarrett *et al.*, 1974) of ketone bodies. Further indirect evidence for insulin's effect on fat metabolism is that diabetic sheep have elevated plasma acetate concentrations

and decreased hind-limb acetate uptake (Jarett *et al.*, 1974).

Short-term regulation of insulin secretion involves modulation by metabolites such as propionate and butyrate (Sasaki *et al.*, 1977), glucose (Anderson, 1974), hormones (including gastrointestinal ones) and the nervous system (McDowell, 1983). The secretion of insulin is also altered with environmental conditions e.g. cold (Sasaki and Weekes, 1986) and physiological state (Metz and Van der Bergh, 1977, Oddy, 1985).

5.1.2.2.2 insulin-like growth factor I (IGF-1)

Insulin like growth factor - I has biological actions very similar to insulin. One feature unique to IGF-1, is that it is a mediator of the physiological effects of growth hormone (reviewed Froesch *et al.*, 1986, Steele and Elasser, 1989). Biological effects (reviewed Steele and Elasser, 1989, Lowe, 1991), include increases in cell proliferation, amino acid and glucose uptake, cellular differentiation, protein synthesis, bone formation and fat synthesis. IGF-1 reduces protein degradation.

IGF-1 is carried through the blood stream by carrier proteins of which one is regulated by GH (Hintz *et al.*, 1981, Schwander *et al.*, 1983). There are two major classes of ovine IGF-1 binding protein fractions with apparent molecular weights of 150 kDa and 40-50 kDa (Hodgkinson *et al.*, 1989). There is further evidence that each of these binding fractions contain multiple species of binding protein (reviewed Hodgkinson *et al.*, 1991). The carrier protein is thought to prolong the half-life of IGF-1 by keeping it inactive while it is carried to its target tissue (Clemmons, 1991). IGF-1 binding proteins can be regulated in response to meals thereby stimulating involvement of IGF-1 in glucose disposal (Yeoh and Baxter, 1988). It has become apparent that IGF-1 may also have several biological actions which are independent of growth hormone (Phillips, 1986).

IGF-1 was first reported as synthesised in the liver (Siess *et al.*, 1965, Schwander *et al.*, 1983). It now appears that many tissues are capable of synthesising IGF-1 (D'Ercole *et al.*, 1980, Binoux *et al.*, 1982). IGF-1 secretion is regulated by several hormones such as growth hormone (Schwander *et al.*, 1983), insulin, corticosteroids, thyroxine and oestrogen (Adamo *et al.*, 1991). In addition, nutrition plays a large role in regulation of tissue and plasma IGF-1 in humans (Phillips, 1986) and in sheep (Hua *et al.*, 1993). IGF-1 levels vary during development and show marked surges around puberty (Gluckman *et al.*, 1979, Roberts *et al.*, 1991).

Research areas of particular interest to the agricultural industry are studies focusing on IGF-1 as a marker of body growth. So far, the results remain inconclusive. Some researchers have concluded that IGF-1 can be used as a predictor of growth performance in cattle (Ringberg Lund-Larsen *et al.*, 1977) and pigs (Ringberg Lund-Larsen and Bakke, 1975), while others question the validity of this approach. For example Gahagan *et al.* (1980) found no difference in IGF-1 between fast and slow growing swine although the lean line had higher insulin concentrations. In addition, infusion of IGF-1 into mini-poodles did not produce increased growth (Guler *et al.*, 1989).

5.1.2.2.3 Cortisol

Glucocorticoids increase glucose concentrations in fed (Reilly and Ford, 1974) and fasted (Bassett, 1968) sheep. Cortisol also plays an important role in the insulin tolerance mechanism: Adrenalectomized sheep showed severe hypoglycaemic signs after insulin injection which could be alleviated by cortisol (Moriya *et al.*, 1985). Cortisol inhibits peripheral glucose utilization and promotes the breakdown of proteins. This releases gluconeogenic amino acids, which, once deaminated, are used as precursors for gluconeogenesis.

Cortisol levels increase markedly in response to climatic and emotional stress. Increases in cortisol levels in sheep have been reported in response to herding (Thurley and McNatty, 1973), pain (Shutt *et al.*, 1987) and wet and cold environments (Panaretto and Vickery, 1970, Reid, 1962). Not surprisingly, adrenaline and noradrenaline, can also increase cortisol levels (McNatty and Thurley, 1973).

5.1.2.2.4 Growth Hormone (Somatotropin)

Growth hormone, produced by the anterior pituitary, stimulates bone and cartilage growth. It also elevates the rate at which fats are utilized, stimulates protein synthesis and nitrogen retention, and elevate blood sugar (reviewed Boyd and Bauman, 1989, Beermann, 1989). Growth hormone promotes weight gain. It is also "diabetogenic" in that its net effect is a decrease in glucose oxidation. It generally promotes protein synthesis and prevents the catabolism of amino acids. Many biological actions of GH are mediated via IGF-1.

The stimulant of growth hormone release (which is a pulsatile process) is growth hormone

releasing factor (GHRH). This factor is produced by the hypothalamus and responds rapidly to a depression in blood glucose levels (Belchetz, 1984). Conversely, somatostatin is a hormone which inhibits the release of growth hormone (reviewed Millard, 1989).

5.1.2.2.5 Melatonin

Melatonin is produced by the pineal gland and is a derivative of tryptophan. The excretion of melatonin follows a circadian rhythm which can regulate the onset of various physiological processes such as moulting. The hormone is thought to mediate reproductive responses in the ewe (Bittman *et al.*, 1983). In sheep the pineal is photo-sensitive, and melatonin levels are highest during the hours of darkness and lowest during daylight (Lincoln *et al.*, 1982). The duration of the elevation of melatonin levels corresponds to the length of the dark period (Almeida and Lincoln, 1982, Bittman *et al.*, 1983).

5.1.2.2.6 Triiodothyronine

T_3 (3,5,3'-triiodothyronine) and T_4 (thyroxine, 3,5,3'5'-tetraiodothyronine) are iodinated derivatives of the amino acid thyronine. They have similar biological actions in mammals (Gorbman, 1978), although T_3 is more active than T_4 in eliciting a response (Hadley, 1988). T_3 can be converted to T_4 extra-thyroidally (Pitt-Rivers *et al.*, 1955, Ingbar and Galton, 1975).

Thyroid hormones have an important role in thermoregulation. Disturbances in thyroid metabolism are often reflected in the basal metabolic rate via an increased rate of oxygen consumption and internal heat production. The release of thyroid hormones is stimulated by cold exposure in many mammals (Hadley 1988, Sterling and Lazarus, 1977, Kennedy *et al.*, 1985). Although it was initially thought that thyroid hormones acted via an uncoupling of oxidative phosphorylation, it is now recognised that their effects on metabolic rate are produced via a stimulation of mitochondrial oxygen consumption and ATP production. This provides the energy required for sodium pumping (Edelman, 1974), a process which liberates a substantial amount of heat and uses 20-40% of cellular energy (Hadley, 1988). Removal of the thyroid disturbs growth in deer (Hadley, 1988, Loudon *et al.*, 1989) and sexual development in sheep (Nicholls *et al.*, 1988).

Regulation of thyroid function (reviewed Hadley 1988) is under the control of the anterior pituitary via thyroid stimulating hormone (TSH). TSH release in turn is under the control of the hypothalamus (via thyroid releasing hormone, TRH).

5.2 Methods

5.2.1 Experimental period and sampling procedure

Since minimum fibre diameter occurs in the wool grown during July-August, the search for markers of wool strength was restricted to this period. Blood sampling occurred before feeding, at fortnightly intervals between June 6th and August 15th. Pasture fed animals were kept in yards for two hours before sampling commenced.

Blood was taken from the jugular vein into vacuated heparinized tubes (10 ml, Venoject, Terumo) using 20 gauge needles (Terumo). Collected blood was stored on ice water until sampling was completed. Plasma was separated by centrifugation at 3000 rpm for 10 minutes.

A blood sample for the measurement of melatonin levels was taken between 10.00-11.00 pm. Vision was aided by the use of red light.

5.2.2 Animal management and fleece measurements

As described in Chapter 4.

5.2.3 Glucose and insulin tolerance tests

The responsiveness and sensitivity of an animal to insulin, can be assessed by the glucose and insulin tolerance tests. These tests involve administering a large dose of glucose or insulin respectively, and changes in plasma glucose are subsequently measured. The shape of the glucose tolerance curve (Figure 5.1) is determined by the capacity of the body to secrete insulin, the rate of insulin catabolism, peripheral tissue sensitivity, the presence of insulin antagonists, and counter regulatory factors, eg glucagon (Linder, 1991). The shape of the insulin tolerance curve, (Figure 5.2) while shaped by similar factors to those mentioned for the glucose tolerance curve, is assumed to be independent of the capacity of the body (pancreas) to secrete insulin. It therefore provides a better indication of peripheral tissue sensitivity.

Glucose tolerance was assessed by the administration of 0.6 ml/kg of prewarmed sterile 50% dextrose through a winged butterfly catheter assembly (Venisystems, Butterfly 19, Abbott Laboratories (N.Z. Limited) Naenae, Lower Hutt). Blood samples were collected at 0, 20, 40, 60 and 90 minutes into vacuated potassium oxalate/sodium fluoride 3 ml tubes (Venoject) using 21 gauge needles (Terumo).

Insulin tolerance was assessed by a single jugular injection of bovine insulin adjusted to be proportional to metabolic liveweight (0.6 IU/$kg^{0.75}$, Gibco, crystalline) using a 0.5 ml syringe with 27 gauge needle (Terumo). This was followed by the collection of venous blood samples at 0,5,8,11,14,17 and 20 minutes into vacuated 3 ml tubes (Venoject) containing potassium oxalate/sodium fluoride using 21 gauge needles (Terumo).

For both glucose and insulin tolerance tests, plasma was separated by centrifugation at 3000 rpm for 10 minutes. Glucose was measured as total reducing sugars using a continuous flow PAHBAH method (Lever, 1977) on Auto Analyzer II modules (Technicon Instruments Corp., New York, USA). A digitizer with an inbuilt regression programme was used on the traces to calculate sample concentration after calibration with standard samples.

T-half values from the glucose tolerance test were obtained using a Genstat program developed by MAF Technology, Lincoln (Appendix IV). T-half represents the time taken to clear half the maximal glucose as estimated from a linear regression on a semi-logarithmic plot. The parameter was calculated as described by Wastney *et al.* (1982). Rate of glucose clearance for the insulin tolerance test was calculated by the general linear models procedure (GLM) of the SAS package (appendix V).

5.2.4 Metabolite assays

Plasma metabolite levels of the blood samples obtained fortnightly (see section 5.2.1) were analyzed at Lincoln University using an Instrumentation Laboratories (IL) Multistat 3 Centrifugal analyzer.

5.2.4.1 Glucose

Plasma glucose was measured using a Boehringer Mannheim kit (Cat No. 263 826) which makes use of the hexokinase / G6P-DH UV method (absorbance measured at 340 nm). In

the glucose and insulin tolerance tests, glucose was measured using the PAHBAH method (section 5.2.7).

5.2.4.2 Urea

Plasma urea was assessed using a BGH Biochemical Company kit (Cat Ref BUNK2) and a UV kinetic method. The reaction utilized involved the hydrolysis of urea by urease to produce ammonia after which it is coupled with α-ketoglutarate and NADH to produce glutamate and NAD^+. The decrease in NADH is measured as a reduced absorbance at 340 nm.

5.2.4.3 Creatinine

Plasma creatinine levels were measured using a Boehringer Mannheim kit (Cat No.124 192). The reaction produced a coloured complex between creatinine and picrate in an alkaline medium. Formation of the complex was measured by a change in absorbance at 520 nm.

5.2.4.4 ß-hydroxybutyrate

An enzymatic colorimetric method employing the 3-hydroxybutyrate dehydrogenase reaction (Boehringer Mannheim kit, Cat No. 907 979) was used to measure ß-hydroxybutyrate. Absorbances were measured at 340 nm.

5.2.5 Hormone assays

5.2.5.1 Insulin

Plasma insulin was measured at Lincoln University using a protocol provided by Sigma with their bovine insulin antiserum (Cat No. I-6136, for a detailed procedure see Appendix I). Modifications from the Sigma protocol included the use of 12 bovine standards in the 0-200 μU/ml range and the counting of radioactivity in the pellet rather than the

supernatant. It was also extremely important that all experimental work was carried out on ice. Samples and standards were incubated with antiserum for five hours before the addition of bovine ^{125}I-insulin. Tracer was prepared using Chloramine T as described by Willis (1990). After the addition of tracer, tubes were incubated overnight and bound and unbound insulin separated using a charcoal solution. Pellets were counted for 10 minutes on an LKB gamma counter. Inter- and intra-assay mean coefficients of variation were 23% and 2.1% respectively. Because of the high inter-assay coefficients of variation only intra-assay comparisons were made. While it is a concern, it is not known why inter-assay variation was so high.

5.2.5.2 Insulin-like growth factor I

Plasma IGF-1 levels were measured by AgResearch Invermay. An automated reversed phase HPLC extraction technique was used to prepare plasma for radioimmunoassay (Moore *et al.*, 1993, Suttie *et al.*, 1993). The intra assay mean coefficient of variation was 8.1%. All samples were assayed in one assay.

5.2.5.3 Triiodothyronine

Plasma T_3 was determined by the Christchurch Medical School by radioimmunoassay according to the method of Saddler and Brownlie (1975). Mean coefficients of variation were 4.5% and 3% for between and within assay variation respectively.

5.2.5.4 Cortisol

Plasma cortisol was measured by Christchurch Hospital according to the method of Lewis *et al.* (1992) using the ELISA technique. The intra mean coefficient of variation was 8.6 %.

5.2.5.5 Growth Hormone

Plasma samples were analyzed by AgResearch Invermay using a standard second antibody competition assay using an ovine kit supplied by NIDDK. The antiserum used, was NIDDK-anti-oGH-2 (AFP-C0123080) and the antigen used, for both standards and iodinations, was NIDDK-oGH-1-5 (AFP-6762B). All samples were analyzed in one assay.

The intra assay coefficient of variation of 7.8%.

5.2.5.6 Melatonin (N-acetyl-5-methoxytryptamine)

Samples were analyzed at Lincoln University using a radioimmunoassay described by Fraser *et al.* (1983). The tracer used, was ^{3}H-melatonin. Ovine anti-melatonin antiserum was obtained from Guildhay Antisera Ltd. (Biochemistry Department, University of Surrey, Guildford, Surrey, GU2 5XH, Great Britain). All samples were analyzed in one assay. The intra assay coefficient of variation was 14.2%.

5.2.6 Statistical analysis of results

Pearson's product-moment correlation coefficients between biochemical markers and fleece data were calculated using the CORR procedure of SAS and the significance of treatment/sex mean differences was calculated using the SAS GLM procedure. The latter uses the method of least squares to fit general linear models (SAS/STAT User Guide Volume 2, 1990).

Simple statistics (means, standard deviations for the calculation of standard errors) were calculated using QUATTRO PRO where this information had not been given by SAS.

5.3 Results

Potential markers of wool strength were assessed during two treatments. These treatments, one involving a difference in management and the other a difference in gender, were chosen to reflect conditions which have traditionally been implicated in affecting wool growth (weather, lactation, feed quality). Live weights were successfully maintained to ± 2 kg from the outset of the experimental phase. This allowed each treatment to be studied independent of changes associated with live weight gain or loss.

To assess their suitability as a marker of wool strength, physiological parameters (hormones and metabolites), were correlated against parameters of wool strength (minimum fibre diameter, staple strength and breeding value for staple strength). Only within treatment comparisons were made.

Between given comparisons (eg. rams vs ewes, indoors vs outdoors, Tables 5.1 and 5.2) were not possible due to confounding factors such as age, sampling procedure, age at shearing and pregnancy. Screening parameter means and SEM values for these groups are presented in tables 5.1 and 5.2.

5.3.2.1 Outdoors

High to moderate correlations were seen for both rams and ewes between plasma insulin and parameters of fleece quality (Table 5.3). For example, the average correlation across weeks between plasma insulin and breeding value for the ewes was highly significant (figure 5.3). The correlation explained up to 72% of the variation in SSBV.

Rams, in particular showed moderate negative correlations between breeding value and glucose (Figure 5.4). As much as 40% of the variation in breeding value could be explained in terms of plasma glucose. On average, 27% of the SSBV variation in rams, and 5% of the SSBV variation in ewes was explained by plasma glucose.

Higher wool strength animals had lower plasma urea levels than the low strength animals (Figure 5.5). Correlations between urea and parameters of wool quality (SSBV, SS and min FD) explained up to 68% of the variation observed (11% on average).

A negative correlation between breeding value and GH was observed in the rams.

No obvious interaction between gender and the correlation between a metabolic marker and wool strength were found. The only exception was that ewes showed higher correlations with plasma insulin and parameters of fleece quality than the rams.

5.3.2.2 Indoors

For the indoor animals, correlative results mostly support those for the outdoors (Table 5.4). For example, there were trends for negative correlations between glucose and parameters of fleece quality, while there were significant positive correlations with insulin. Correlations observed with urea were both positive and negative in sign thereby hindering interpretation. A finding unique to the indoor group was an increase in glucose tolerance, yet a decrease in the response to insulin (Figure 5.7) as parameters of wool quality improved.

		OUTDOOR (n = 24)		INDOOR (n = 38)	
		Mean	**SEM**	**Mean**	**SEM**
SSBV N ktex^{-1}		2.07	1.422	-0.45	0.877
SS N ktex^{-1}		50.14	4.039	68.82	3.000
FD µm		31.64	0.836	31.96	0.490
LWT kg		65.00	1.212	50.22	0.900
Glucose	(week 2)	63.04	1.314	56.34	1.010
mg dL^{-1}	(week 4)	63.79	1.316	62.95	1.253
	(week 6)	64.67	1.592	69.58	0.823
	(week 8)	72.92	2.930	54.24	0.644
Average		**66.10**	**1.344**	**58.28**	**0.645**
Urea	(week 2)	43.74	2.149	44.69	2.207
mg dL^{-1}	(week 4)	42.25	2.349	45.92	2.359
	(week 6)	63.13	1.667	33.25	0.651
	(week 8)	62.50	1.642	24.89	0.827
Average		**52.91**	**1.196**	**37.18**	**0.936**
ß-OH butyrate	(week 2)	2.95	0.261	3.69	0.355
mg dL^{-1}	(week 4)	3.33	0.246	3.36	0.176
	(week 6)	2.96	0.218	3.11	0.157
	(week 8)	3.71	0.286	4.14	0.178
Average		**3.24**	**0.134**	**3.57**	**0.123**
Creatinine	(week 2)	1.08	0.027	1.02	0.017
mg dL^{-1}	(week 4)	1.13	0.023	1.09	0.019
	(week 6)	1.00	0.020	1.01	0.016
	(week 8)	1.06	0.026	0.97	0.015
Average		**1.06**	**0.017**	**1.02**	**0.011**
Insulin	(week 2)	12.07	1.437	13.79	1.012
µU ml^{-1}	(week 4)	13.04	1.622	13.87	1.300
	(week 6)	8.64	0.648	20.57	2.578
	(week 8)	19.52	3.665	16.66	1.266
Average		**13.32**	**1.351**	**16.18**	**1.105**
T-half min		100.83	14.597	139.09	9.910
Insulin tolerance (glucose cleared mgdL^{-1} h)		77.02	3.485	66.56	2.025
IGF-1 ng ml^{-1}		263.65	10.478	339.41	9.218
GH ng ml^{-1}		6.37	1.958	9.95	1.537
T3 nmol L^{-1}		0.66	0.038	0.89	0.043
Cortisol nmol L^{-1}		31.50	4.615	15.42	0.633

Table 5.1: Screening parameter means of indoor and outdoor rams. SEM = standard error of the mean.

		RAMS (n = 24)		EWES (n = 24)	
		Mean	**SEM**	**Mean**	**SEM**
SSBV N ktex^{-1}		2.07	1.422	0.11	1.357
SS N ktex^{-1}		50.14	4.039	49.54	4.450
FD µm		31.64	0.836	30.29	0.845
FWT kg		5.44	0.141	3.92	0.105
LWT kg		65.00	1.212	60.27	1.815
Glucose	(week 2)	63.04	1.314	63.75	1.814
mg dL^{-1}	(week 4)	63.79	1.316	63.67	3.069
	(week 6)	64.67	1.592	63.75	1.533
	(week 8)	72.92	2.930	72.33	3.917
Average		**66.10**	**1.344**	**65.88**	**1.613**
Urea	(week 2)	43.74	2.149	43.66	2.352
mg dL^{-1}	(week 4)	42.25	2.349	45.52	2.858
	(week 6)	63.13	1.667	59.01	2.610
	(week 8)	62.50	1.642	50.38	1.201
Average		**52.91**	**1.196**	**50.37**	**1.704**
ß-OH butyrate	(week 2)	2.95	0.261	3.52	0.344
mg dL^{-1}	(week 4)	3.33	0.246	3.45	0.275
	(week 6)	2.96	0.218	4.37	0.446
	(week 8)	3.71	0.286	7.10	0.645
Average		**3.24**	**0.134**	**4.56**	**0.252**
Creatinine	(week 2)	1.08	0.027	1.10	0.027
mg dL^{-1}	(week 4)	1.13	0.023	1.20	0.036
	(week 6)	1.00	0.020	0.96	0.020
	(week 8)	1.06	0.026	1.04	0.024
Average		**1.06**	**0.017**	**1.07**	**0.016**
Insulin	(week 2)	12.07	1.437	13.51	1.868
µU ml^{-1}	(week 4)	13.04	1.622	12.21	1.272
	(week 6)	8.64	0.648	12.60	1.148
	(week 8)	19.52	3.665	12.60	1.390
Average		**13.32**	**1.351**	**12.68**	**1.197**
T-half min		100.83	14.597	112.36	7.720
Insulin tolerance (glucose cleared mgdL^{-1} h)		77.02	3.485	70.81	3.744
IGF-1 ng ml^{-1}		263.65	10.478	191.32	6.809
GH ng ml^{-1}		6.37	1.958	0.18	0.118
T3 nmol L^{-1}		0.66	0.038	0.82	0.048
Cortisol nmol L^{-1}		31.50	4.615	55.00	4.127

Table 5.2: Screening parameter means of outdoor rams and ewes. SEM = standard error of the mean.

	SSBV		SS		minFD	
	♂	♀	♂	♀	♂	♀
Glucose						
Week 2	0.02	-0.17	0.16	0.30	0.13	0.06
Week 4	-0.43**	-0.28	-0.24	-0.17	-0.17	-0.26
Week 6	-0.25	-0.23	0.05	-0.03	0.12	-0.01
Week 8	-0.64****	0.02	-0.57***	0.32	-0.40**	0.17
Average	**-0.52*****	**-0.22**	**-0.32**	**0.19**	**0.19**	**-0.00**
Urea						
Week 2	-0.49**	-0.37*	-0.46**	-0.44**	-0.39*	-0.39*
Week 4	-0.68****	-0.42**	-0.61***	-0.46**	-0.52***	-0.37*
Week 6	0.35*	0.09	0.40**	-0.19	0.13	0.04
Week 8	0.17	0.06	0.06	0.27	-0.14	0.18
Average	**-0.37***	**-0.26**	**-0.35***	**-0.37***	**-0.43***	**-0.24**
ß-OH butyrate						
Week 2	-0.17	-0.28	-0.22	-0.10	0.16	-0.08
Week 4	0.13	0.03	-0.17	0.22	0.23	0.17
Week 6	0.02	-0.10	-0.02	-0.09	-0.04	0.03
Week 8	-0.10	-0.01	0.21	-0.57**	0.02	-0.09
Average	**-0.07**	**-0.13**	**-0.08**	**-0.38**	**0.18**	**-0.05**
Creatinine						
Week 2	0.22	-0.23	0.26	-0.32	0.04	-0.27
Week 4	-0.23	-0.26	-0.08	-0.40*	-0.20	-0.34
Week 6	0.14	0.25	0.26	0.03	0.09	0.36
Week 8	0.40**	0.22	0.26	0.32	0.19	0.52***
Average	**0.20**	**-0.09**	**0.25**	**-0.26**	**0.04**	**0.01**
Insulin						
Week 2	0.77****	0.80****	0.57***	0.48**	0.52***	0.61***
Week 4	0.65****	0.74****	0.51**	0.43**	0.45**	0.68***
Week 6	0.16	0.74****	0.18	0.21	0.26	0.56***
Week 8	0.02	0.65****	-0.19	0.49**	0.00	0.48**
Average	**0.43****	**0.85*****	**0.20**	**0.48****	**0.31**	**0.68*****
T-half	-0.28	-0.29	-0.18	0.16	-0.20	-0.01
Insulin tolerance	0.22	-0.11	0.37	0.11	0.16	-0.16
IGF-1	-0.24	-0.18	-0.12	0.22	0.07	0.20
GH	-0.50**	-0.20	-0.29	0.31	-0.05	0.31
T3	0.12	-0.12	0.05	0.26	-0.12	0.05
Cortisol	-0.06	0.03	0.17	0.09	-0.01	-0.18

Table 5.3: Pearson correlation coefficients between screening parameters and determinants of staple strength for the outdoor rams (n = 24) and ewes (n = 24).
Levels of significance are as follows: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$.

	SSBV	SS	minFD
Glucose			
Week 2	-0.18	-0.30*	-0.23
Week 4	-0.10	0.15	0.01
Week 6	-0.48***	-0.23	0.05
Week 8	-0.29*	-0.50***	-0.24
Average	**-0.35****	**-0.25**	**-0.13**
Urea			
Week 2	-0.31*	-0.16	-0.23
Week 4	-0.38**	-0.14	-0.20
Week 6	0.34**	0.33**	0.37**
Week 8	-0.09	0.09	0.36**
Average	**-0.38****	**-0.10**	**-0.12**
ß-OH butyrate			
Week 2	0.03	0.02	0.14
Week 4	0.21	0.16	0.17
Week 6	-0.15	-0.17	0.01
Week 8	0.12	0.07	0.04
Average	**0.09**	**0.04**	**0.18**
Creatinine			
Week 2	-0.08	-0.07	-0.35**
Week 4	-0.25	0.17	-0.09
Week 6	-0.05	-0.10	0.15
Week 8	-0.03	-0.07	0.26
Average	**-0.14**	**-0.01**	**-0.03**
Insulin			
Week 2	0.23	0.16	0.18
Week 4	0.34*	0.43***	0.49***
Week 6	-0.12	0.02	-0.06
Week 8	0.46***	0.41**	0.42**
Average	**0.20**	**0.28***	**0.27**
T-half	0.08	-0.42**	-0.35**
Insulin tolerance	-0.36**	-0.14	0.02
IGF-1	0.06	-0.03	0.03
GH	-0.07	-0.09	-0.04
T3	-0.01	0.10	0.26
Cortisol	0.02	-0.02	0.07
Melatonin	-0.09	-0.12	-0.12

Table 5.4: Pearson correlation coefficients between screening parameters and determinants of staple strength for the indoor rams (n = 38). The level of significance is as follows: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, ****, $P < 0.0001$.

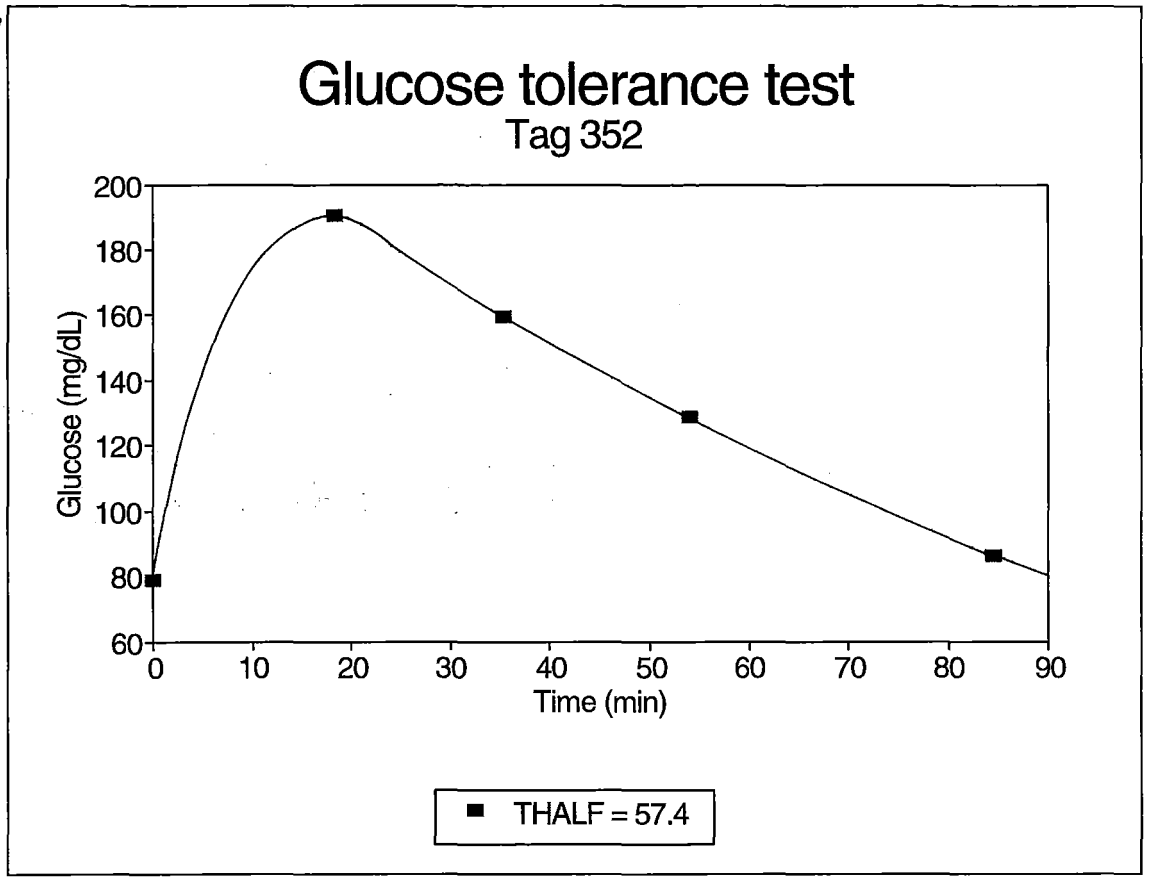


Figure 5.1: Typical glucose tolerance curve.

Initially, a steep rise in plasma glucose occurs as the result of administration of an intravenous glucose load. The body responds by moving glucose into the tissues in order to maintain glucose homeostasis. The response is measured in terms of T-half, the time taken for half the administered glucose load to clear from the bloodstream.

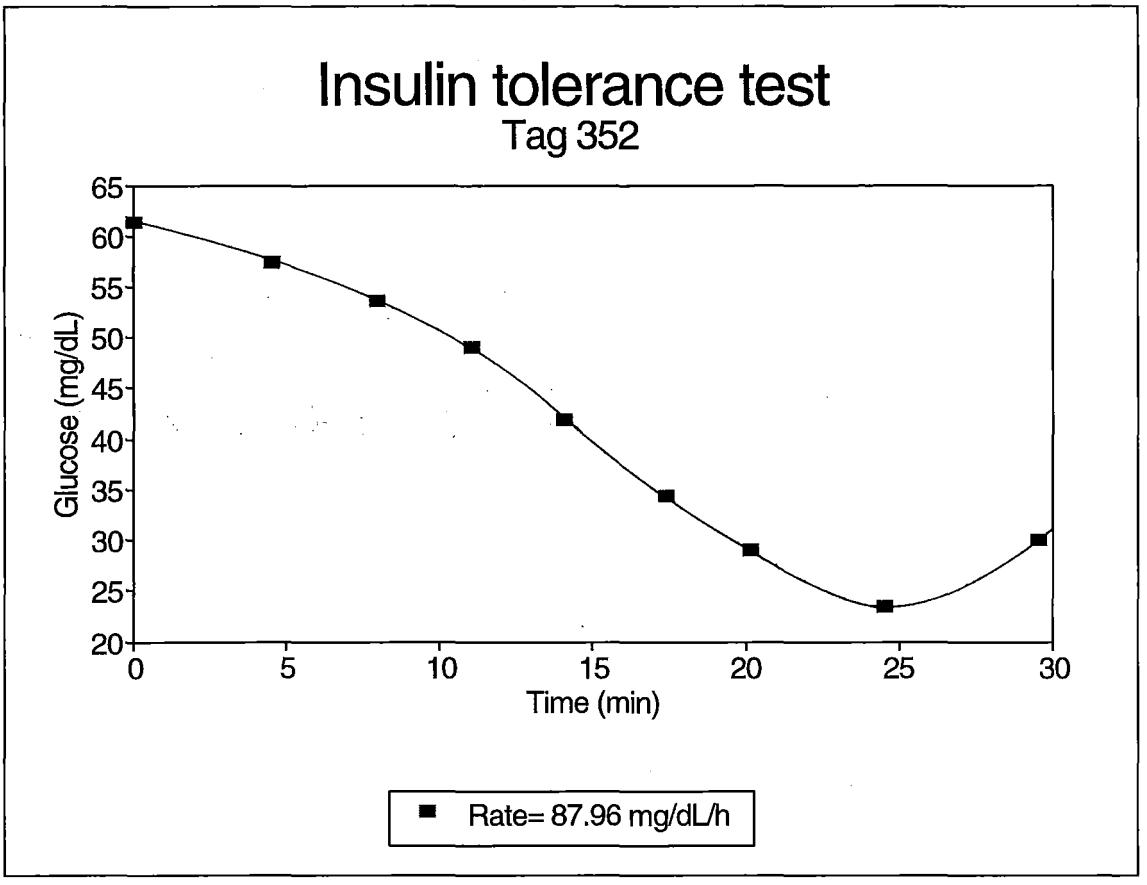


Figure 5.2: Typical insulin tolerance curve.

Plasma glucose levels decline steadily as glucose is taken up into the tissues in response to insulin. Insulin tolerance is assessed as the rate of glucose clearance in the 0-30 minute time interval after administration of an intravenous insulin dose.

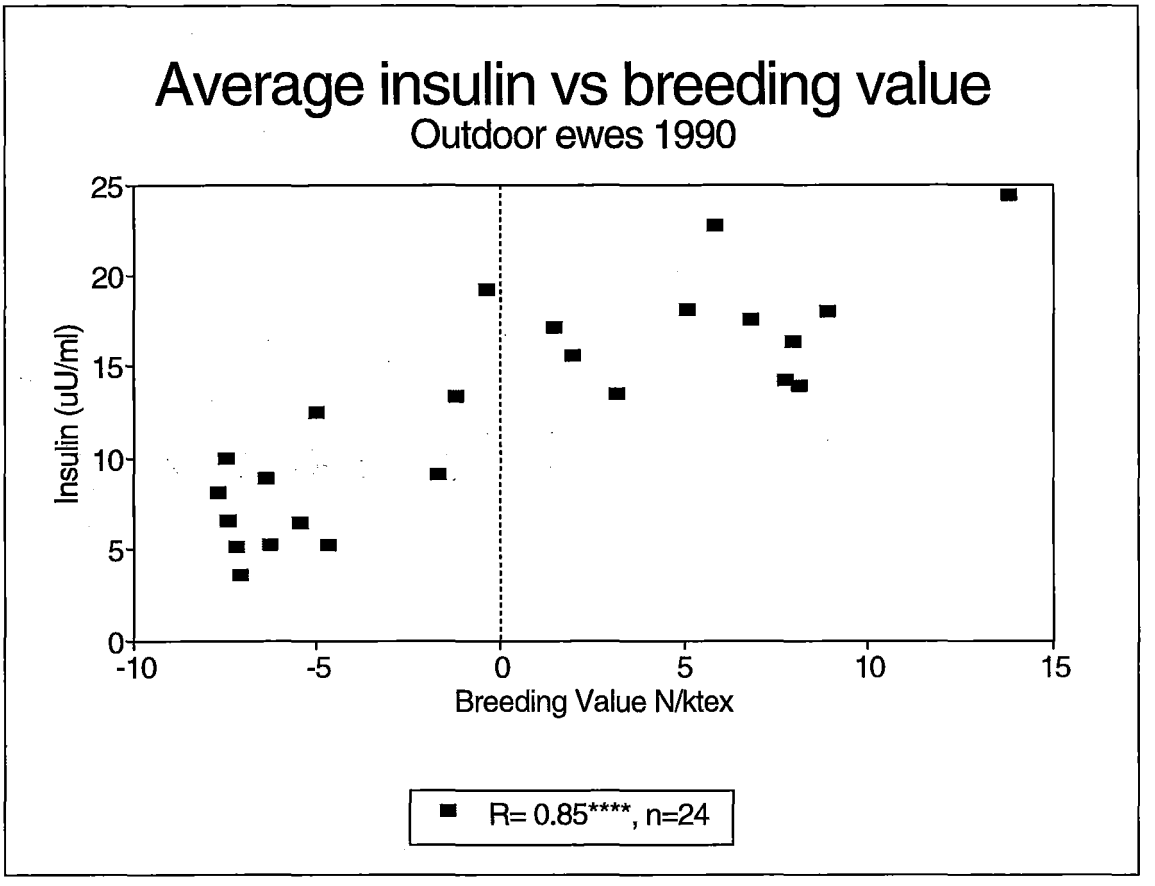


Figure 5.3: Scatterplot showing the positive association between plasma insulin and the breeding value for staple strength.

The insulin values shown represent an average for each animal across 4 weeks. The vertical dotted line represents the transition between animals likely to produce progeny of low staple strength and high staple strength. The correlation coefficient is significant ($p < 0.0001$).

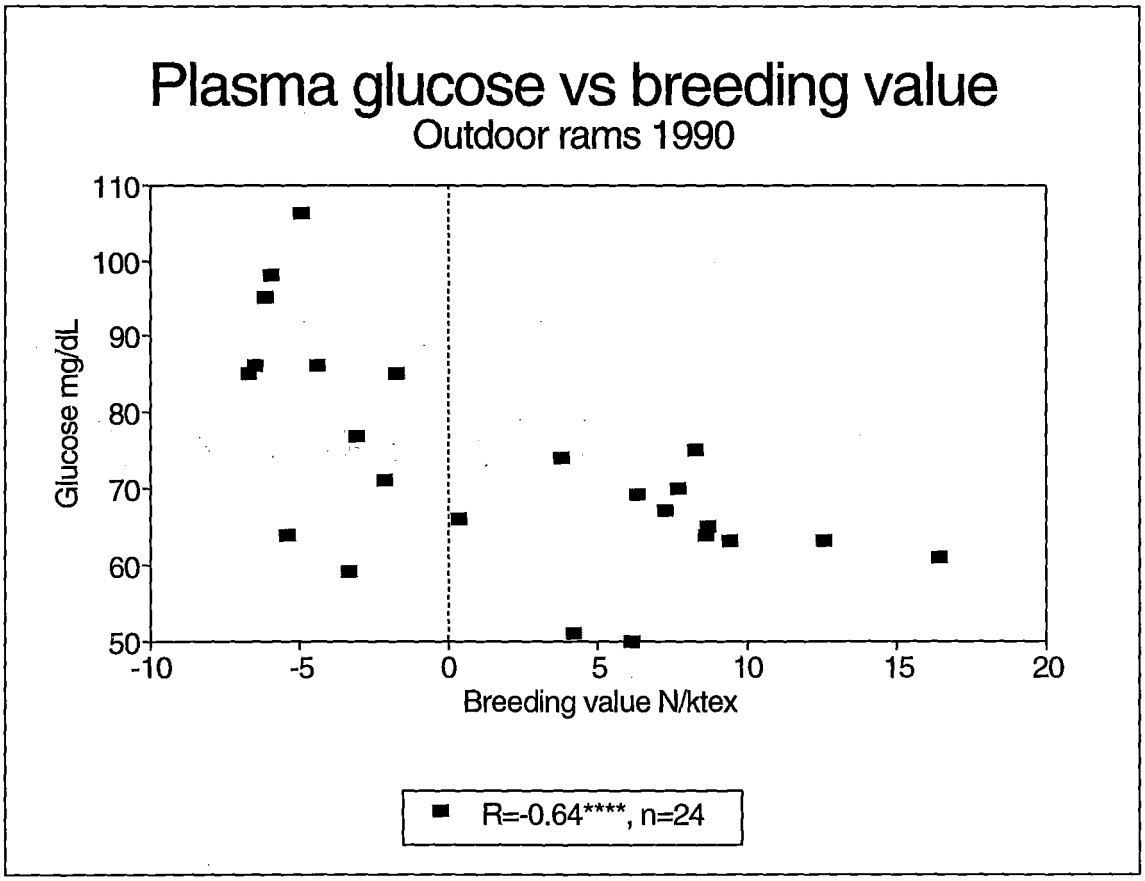


Figure 5.4: Scatterplot illustrating the negative association between plasma glucose and breeding value for staple strength.

The correlation coefficient is significant ($p < 0.0001$).

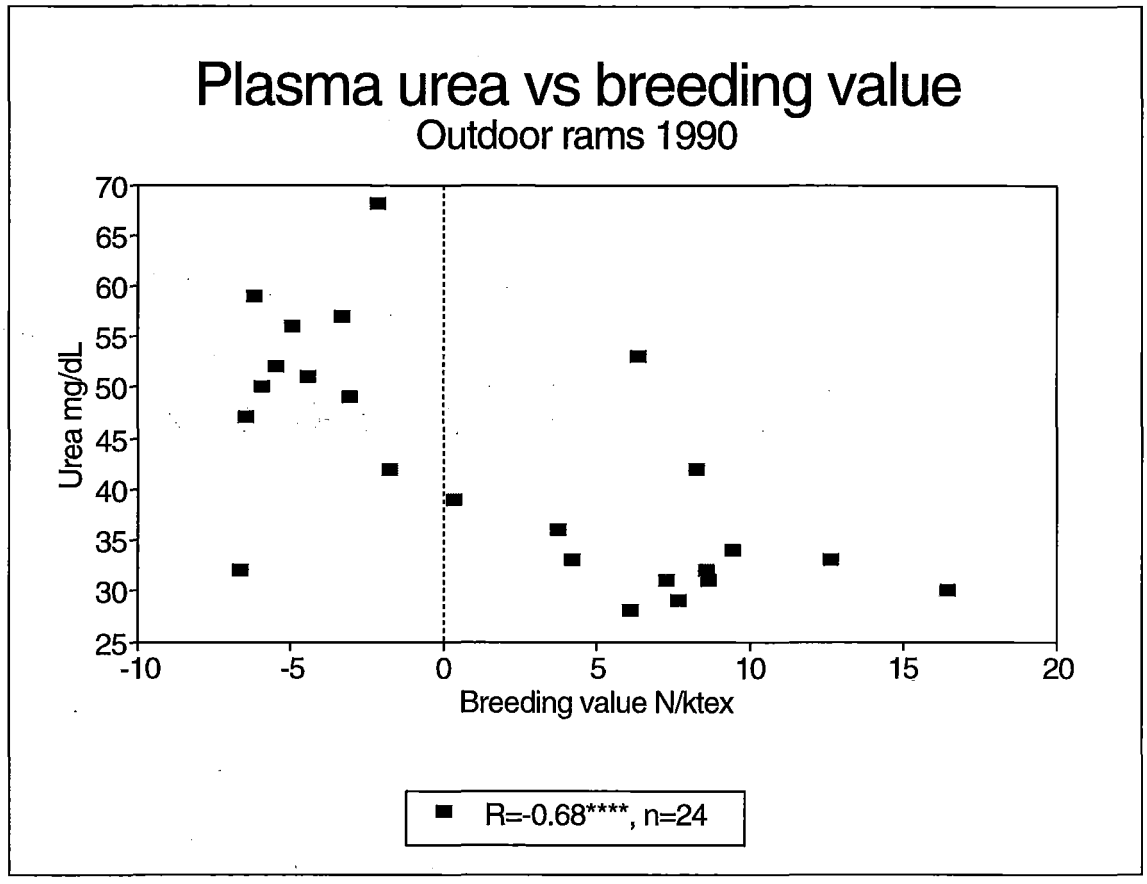


Figure 5.5: Scatterplot of the negative association between plasma urea and breeding value for staple strength.

The correlation coefficient is significant ($p < 0.0001$).

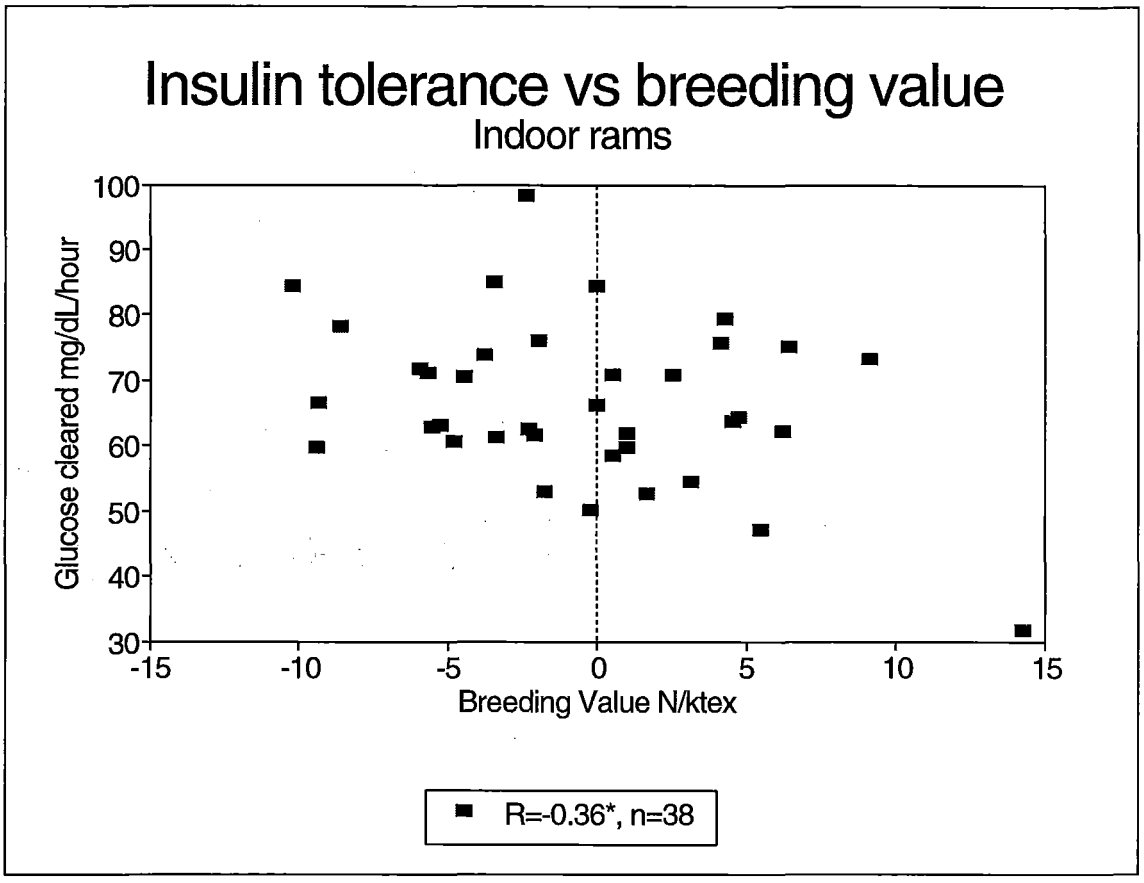


Figure 5.6: Scatterplot of insulin tolerance graphed against the breeding value for staple strength.

Insulin tolerance represented as the rate of glucose clearance after an insulin injection and is a function of peripheral sensitivity to insulin. The correlation coefficient shown is significant ($p < 0.05$). While the correlation is strongly influenced by the animal with the highest breeding value, its data was included because this animal consistently had unusual data with regard to insulin status and his fleece was of superior staple strength thereby fitting the model proposed. Animals such as these are extremely useful when seraching for genetic markers since they are likely to be homozygous, for such a locus. The correlation between insulin tolerance and breeding value is not significant however (R=0.18) without inclusion of this animal.

5.4 Summary of results

- Staple strength, fibre diameter and SSBV were not affected by gender.
- Fleece weights and live weights were heavier for the rams.
- Sheep with greater wool strength were hypoglycaemic and hyperinsulinaemic and this was sometimes associated with changes in glucose clearance rates.
- The association between wool strength and insulin status occurred across gender and management regime to varying degrees. The animals maintained on pasture showed much higher and consistent correlations between staple strength and plasma insulin.

5.5 Discussion

Considerable variation in staple strength and breeding value for staple strength existed between animals within the same treatment group thereby making the data well suited to correlative analysis.

In the outdoor group, higher wool strength animals had lower plasma urea. Changes in plasma urea have not been reported previously as directly associated with staple strength, but indirect evidence for the correlation is provided by McCutcheon *et al.*, 1987 and Thomson *et al.*, 1989. These researchers studied the effect of genetic selection for greasy fleece weight on rumen metabolism, apparent digestibility, and nitrogen metabolism in Romney sheep. They found that high fleece weight animals had a greater voluntary feed intake as well as showing differences in the rate of particulate matter passage (Thomson *et al.*, 1989). High fleece weight lines had lower plasma urea concentration and faster creatinine clearance. Since fleece weight is dependent on fibre diameter, these results may explain why staple strength (also dependent on fibre diameter) is correlated to plasma urea.

Urea and staple strength associations were not observed for the indoor group. It is not known why this correlation is not consistent across treatments but it could be that it is necessary for animals to express differences in appetite if correlations between urea and staple strength are to occur. Differences in appetite were allowed to be expressed in the outdoor animals and consequently lighter animals may have had a proportionally higher intake than heavier animals. If these lighter animals were metabolically inefficient and did not gain live weight, then mean live weight for the group would have remained unaltered. This could provide a mechanism by which metabolically "inefficient" animals increase wool growth when they partition nutrients to the wool follicle instead of muscle or adipose tissue. Why this change in partitioning comes to be associated with changes in plasma urea is not known. However, the wool follicle-fibre complex produces a tissue which is inert and excreted outside the body (wool). Once synthesised therefore, it is unlikely to be involved in further transamination reactions and amino acid catabolism. This may have been responsible for the reduction in plasma urea.

Urea results support the hypothesis that differences in protein turnover occur as wool strength increases - possibly in response to insulin. Infusion of insulin into pigs using a euglycaemic clamp, decreased ureaN of the urine (Fuller *et al.*, 1977). This suggests that increases in insulin, not decreases in plasma glucose, are responsible for the change in

plasma urea. In sheep, insulin infusion decreases urea and amino nitrogen (Call *et al.*, 1972).

A major finding in this work was that wool strength is associated with higher plasma insulin. Correlations were often high, highly significant and occurred in both indoor and outdoor groups. This would appear to rule out the possibility that insulin's sole involvement as a marker of wool strength is as a covariate of appetite, since appetite was not expressed by the indoor animals. A positive correlation between insulin and clean wool growth has been reported previously (Oddy, Wallace and Jones unpublished in Oddy and Lindsay, 1986). The correlation raises the possibility that the rise in plasma insulin favours the partitioning of nutrients to the wool follicle. Insulin previously has been shown previously to have such an effect with regard to other tissues in sheep (Oddy *et al.*, 1987).

The relationship between plasma insulin and wool strength was most pronounced in the outdoor animals, and in ewes suggesting that the extent of the insulin/staple strength association could depend on appetite (intake per body weight) or physiological status (pregnancy). Pregnancy has been reported as reducing the availability of glucose to the ewe (Oddy *et al.*, 1985), as well as decreasing insulin sensitivity of some metabolic processes (Metz and Van den Bergh, 1977).

The correlation between staple strength and insulin status could potentially be mediated by simultaneous changes in glucose and amino acid metabolism. With respect to the ovine hind-limb insulin increased hind-limb muscle glucose uptake. Insulin had no significant effect on whole-body or hind-limb muscle of fed lambs. In fasted lambs however, insulin reduced both protein synthesis and degradation (Oddy *et al.*, 1989).

This study suggests that high wool strength animals show sign of mild hypoglycaemia compared to low wool strength animals. In addition, the indoor animals showed a significant decrease in tissue sensitivity to insulin as wool strength increased. Hypoglycaemia in conjunction with insulin resistance is an unusual observation. To explain how this situation may arise, it is important to stress that skin is responsible for significant parts of whole body metabolism (Harris *et al.*, 1989). Furthermore, 80-85 % of glucose utilization in sheep is insulin independent (Janes *et al.* 1985) and only 8-14% of this insulin independent glucose uptake is thought to be used by the CNS (Janes *et al.*, 1985). Given that the uptake of glucose in sheep is greater for skin than for resting muscle (25 versus 3-15 μmol/min/kg, Harris and Lobley 1991) and acetate uptake into the resting hind limb exceeds the net uptake into skin by a factor of two (16-25 versus 8 μmol/min/kg, Jarrett *et al.* 1976), it is conceivable that an increase in peripheral tissue

insulin resistance could partition glucose to the skin where it is able to be taken up in an insulin independent manner.

The apparent changes in protein and energy metabolism of high wool strength animals suggests that high and low wool strength animals differ in the metabolic activity of a tissue large enough to reflect these differences in whole body metabolism. Such a tissue could potentially be skin. At a cellular level, changes in partitioning may arise within the wool follicle via differences in insulin receptor sensitivity or capacity, or alternatively through differences involving the mechanism of glucose uptake (glucose transporters).

Scatchard analysis of insulin receptors in skin

6.1 Introduction

Differences in insulin kinetics between individuals are only rarely caused by differences in the rate of insulin secretion by the pancreas. For example, with respect to diabetes a lack of insulin *per se* does not represent the major disease-related causative factor. Instead the defect appears to be somewhere in the complex chain of reactions whereby insulin activates its target cells (Hollenberg, 1985). Many such defects can result in insulin resistance, insulin insensitivity and insulin unresponsiveness which apart from causing diabetes are known to be at least in part responsible for a number of minor conditions such as hirsutism, *acanthosis nigricans* (Geffner and Golde, 1988), Laron dwarfism, obesity and Cushing's syndrome (Kann, 1978). The insulin receptor is widely implicated in causing many diseases of insulin action.

The physiological state of an individual can also produce marked changes in insulin receptor binding. Some women show reduced insulin binding with pregnancy (Damm *et al.*, 1993). The effect is called gestational diabetes. Furthermore, insulin binding is increased during the luteal phase of the menstrual cycle (Strowitzki *et al.*, 1993). In sheep, changes associated with pregnancy include promotion of lipogenesis via an increase in the number of high-affinity insulin receptors, a decrease in the negative cooperativity phenomenon, and an increase in maximal stimulation of lipogenesis in response to insulin (Guesnet *et al.*, 1991).

Insulin receptor binding can also vary with developmental stage and age. Alexandrides *et al.*, (1989), have shown that the receptors for IGF-1, IGF-II and insulin, are differentially expressed in rat skeletal muscle, while Bolinder *et al.*, (1983) have shown that insulin sensitivity of adipose tissue decreases with age as the result of insulin receptor down-regulation. However, it has been shown that in rats (Olefsky and Reaven, 1975) and humans (Boden *et al.*, 1993, Kohrt *et al.*, 1993) that age related insulin resistance does not occur if obesity is prevented. For example, in Type II diabetic patients, insulin resistance may be the result of a defective insulin receptor (Accili *et al.*, 1989, Cocozza *et al.*, 1992) or a decrease in the number of receptors (reviewed Lonnroth, 1991, Moller and Flier, 1991).

6.1.1 Heterogeneity of insulin receptors between and within tissues

The insulin receptor is a 350 kD glycoprotein consisting of 125-135 kD α (Jacobs *et al.*, 1977) and 95kD ß subunits (Massague *et al.*, 1980). These subunits are assembled as a α2ß2 tetramer to form the mature receptor. Disulfide bonds hold the α and ß subunits together (Massague *et al.*, 1981). The α subunits of the receptor protrude on the external cell surface whilst the ß subunits span the whole membrane to occupy the intra- and extra-cellular surfaces simultaneously.

In humans, there are two insulin receptor isoforms which are derived from alternative splicing. The two isoforms differ by the absence or presence of a 12 amino acid region encoded by exon 11. The relative splicing into these two forms is tissue specific. For example, althought the majority of insulin receptors contain exon 11 in all tissues, liver tissue has a lower percentage of RNA molecules without exon 11 than muscle and adipose tissue (Norgren *et al.*, 1994). The 11- isoform is rare and has a high affinity for insulin (McClain, 1991, and Mosthaf *et al.*, 1993). This high affinity receptor isoform may be reduced in muscle from diabetic patients although there is some controversy regarding this issue (Anderson *et al.*, 1993, Norgren *et al.*, 1993, 1994). The two isoforms differ in their ability to bind IGF-1 and their susceptibilities to down-regulation in response to insulin (Yamaguchi *et al.*, 1993). Ligand-induced receptor internalization rates also differed (see Yamaguchi *et al.*, 1993).

Subtle differences in the behaviour of insulin receptors between different tissues exist. For example, rat hepatocytes and adipocytes have different affinities for B26 monoiodoinsulin (Podlecki *et al.*, 1983). Similarly, Breiner *et al.* (1993), while comparing insulin binding between brain, spleen, adipocyte and liver tissues, found that insulin binding in the presence of an agonist differed between tissues. Insulin binding by spleen tissues was different from that in adipocytes, brain and liver tissues. Brain and adipocyte insulin receptors were found to have smaller α subunits. Interestingly, differences in binding could not be accounted for by the relative proportions of the two receptor isoforms. Insulin receptors of the brain show a lesser degree of glycosylation (Heidenreich *et al.*, 1983). This may therefore be the reason for the different binding activity reported in this tissue (Kahn, 1976, Heidenreich *et al.*, 1983).

6.1.2 Insulin receptor binding

The relationship between the fraction of receptors occupied and maximum response to

insulin is not straight forward. For example, maximal stimulation occurs well below maximum occupancy (Kono and Barnam, 1971, Olefsky, 1976). Glucose metabolism in adipocytes is stimulated maximally when only approximately 2.4% of the total insulin receptors on the cells are occupied (Kono and Barnam, 1971). The excess of receptors is presumed to render the cell highly sensitive to insulin (the spare receptor theory). However, the spare receptor theory may not always hold. There is evidence for a lack of spare high-affinity receptors in muscle (Guma *et al.*, 1993) and liver tissues (Menuelle and Plas, 1981).

Binding of insulin produces a conformational change in the receptor (Pilch and Czech, 1980) thereby initiating a cascade of events to produce a downstream biological response. It is thought this conformational change activates an intrinsic tyrosine kinase activity which subsequently brings about the autophosphorylation of specific tyrosine residues on the receptor (Kahn and White, 1988, Haring, 1991). Once phosphorylated, the receptor is unresponsive to insulin and behaves similarly whether its ligand is attached or not (Rosen et al, 1983).

The role of autophosphorylation and tyrosine kinase activity of the insulin receptor may not be crucial in producing some of the biological responses associated with insulin. Mutant insulin receptors which lack a carboxy terminal, the site of autophosphorylation and tyrosine kinase activity, can still phosphorylate endogenous substrates (Yamamoto-Honda *et al.*, 1993, Sasaoka *et al.*, 1993). Glucose uptake and thymidine incorporation were also similar or were sometimes increased. Internalization of the receptor was decreased however (Yamamoto-Honda *et al.*, 1993). Interestingly, removal of the carboxy-terminal of the B-subunit increases receptor signalling. For example, deletion of 90% of the cytoplasmic domain significantly increased the phosphorylation of selected target proteins, glucose incorporation into glycogen and thymidine incorporation (Sasaoka *et al.*, 1993).

The kinase activity of the insulin receptor is thought to initiate the phosphorylation of serine residues on endogenous proteins. Serine phosphorylation is a common mechanism by which enzymes are activated and serine kinases can regulate protein function directly by phosphorylation or indirectly by activating specific phosphatases (Denton, 1990). Consequently, a major goal of current research is to identify signalling intermediates which act as adaptors between tyrosyl and seryl phosporylative pathways. IRS-1 (pp185) is an example of a protein whose tyrosyl residues appear to be phosphorylated by the activated insulin receptor kinase (Yamamoto-Honda *et al.*, 1993). Once IRS-1 is phosphorylated on specific tyrosine residues, these phosphorylated sites associate with cellular domains in target proteins which contain SH2 or SH3 (src homology) domains. These SH domains allow an enzyme to be 'plugged in' to a number of different upstream

activators of which IRS-1 is one. A growing number of SH2 and SH3 domains have been identified in target proteins of insulin action and it is suspected that IRS-1 has the potential to interact with many of them. Examples include growth factor receptor bound protein 2 (Egan *et al.*, 1993) and phosphatidylinositol-3 kinase (PI-3 kinase, Hayashi *et al.*, 1993, Skolnik *et al.*, 1993). Other molecules potentially involved in the generation of insulin effects include pp120 (Ecto-ATPase, Taylor *et al.*, 1991), pp15 (adipocyte homologue of myelin P protein, Taylor *et al.*, 1991), mitogen activated protein kinase (MAP kinase, Taylor *et al.*, 1991), the serine kinase *raf-1* kinase (Denton, 1990), S6 kinase (Denton, 1990, Saltiel, 1990) and protein kinase C (Chin *et al.*, 1993).

Research has recently focused on the idea that *ras* protein (a member of the *ras* oncogene family) may serve as a downstream control point for insulin action from which the various signalling pathways branch off. This is because *ras* is involved with many of the biological activities associated with tyrosine kinases such as the insulin receptor. Kozma *et al.* (1993) have shown that activated *ras* mimics the actions of insulin on membrane trafficking of glucose transporters. Apart from an effect on glucose transport, insulin induced activation of extracelluar signal-regulated kinases, also known as mitogen-activated protein kinases (MAPs) is thought to be mediated by *ras*. MAP kinases, like IRS-1, are thought to be activated by tyrosine phosphorylation (e.g. by the insulin receptor) and are consequently able to bring about the serine and threonine phosphorylation of other proteins (e.g. glycogen synthase, glycogen phosphorylase, pyruvate dehydrogenase and S6 kinase, Taylor *et al.*, 1991). One of the downstream events which occurs as the result of insulin action is an exchange of *ras*-bound GDP for ATP (i.e. phosphorylation of *ras* protein, Yonezawa *et al.*, 1994). Activation of *ras* is enhanced by the presence of Grb2, which has to have its SH2 and SH3 domains intact for the stimulation to occur (Skolnik *et al.*, 1993). Grb2 and PI kinase SH2 domains bound to IRS-1 also contain a Sos (son of sevenless) product which is a putative guanine nucleotide exchange factor. In this context Grb2 functions as an adaptor protein between other proteins containing SH2 and SH3 domains. It has therefore been suggested that *ras*-GDP is phosphorylated by a IRS-GRB2/PI kinase-Sos complex (Baltensperger *et al.*, 1993).

Secondary messengers other than phosphorylated proteins may also exist. Calcium has been suggested as a mediator of cellular responses to insulin since the insulin receptor has a calmodulin binding site (McDonald *et al.*, 1984). Insulin also promotes Ca^{++} binding to adipocytes and reduces activity of the high affinity Ca^{++} Mg++ ATPase (McDonald and Pershadsingh, 1985). Other mechanisms include those which propose an increase in cellular pH, thereby altering the activity of several ion pumps and precipitating a response to insulin (Moore, 1985). Insulin induces hyperpolarization of its main target tissues (increases the electric potential across the cell membrane). This phenomenon has been

associated with an increase in glucose transport and the release of substances from the membrane which may also act as secondary messengers (Zierler, 1985).

After ligand binding the receptor is internalized by an endocytic process (Posner et al, 1981) which decreases the number of receptors on the cell surface. Internalization may down-regulate the receptor and decrease the sensitivity of the cell to high concentrations of plasma insulin (Gavin *et al.*, 1974, Marshall and Olefsky, 1980). Receptor internalization appears to be an ongoing process which is accelerated by insulin binding. Once internalized, the receptor is either stored, degraded or returned to the cell surface. The balance of these events is specific to cell type (Heidenreich and Olefsky, 1985).

Some evidence suggests that the internalized receptor may be able to elicit a biological response itself. Payne *et al.* (1992) have shown binding of insulin to ovine liver nuclei and similar studies by Goldfine *et al.* (1977) on rats included a discussion on the biological significance of this phenomenon and suggested that after internalization the insulin molecule is responsible for acting on the nucleus to instigate RNA/DNA synthesis. Intracellular insulin receptors have also been reported on the Golgi body (Posner *et al.*, 1981) and nuclear membranes (Goldfine *et al.*, 1977).

6.1.3 Regulation of insulin receptor binding

The insulin receptor can be regulated at several levels. Chronic exposure to insulin (5-16 hours) decreases the concentration on insulin receptors found on the cell surface (down-regulation, Gavin *et al.*, 1974). However, acute exposure of rat adipocytes to insulin appears to have the opposite effect. Rat adipocytes pre-incubated with insulin showed a rapid energy dependent increase in cell surface binding of insulin (Eriksson *et al.*, 1992). Of relevance to the work outlined in this chapter is that insulin receptors in human fibroblasts are down-regulated by insulin (Baldwin *et al.*, 1981, Verrando *et al.*, 1985). Down-regulation is presumably achieved by endocytosis of the receptor after which it is either degraded (Gavin *et al.*, 1974) or recycled to the cell surface (Marshall and Olefsky, 1983). Down-regulation of the insulin receptor by insulin may not occur solely via an internalization mechanism. Okabayashi *et al.* (1989) have shown that receptor down-regulation is associated with a 50% reduction in insulin receptor mRNA levels. Receptor degradation was also increased.

Changes in receptor affinity have also been observed thereby providing a means of receptor regulation. Fasted rats show an increase in overall binding affinity for insulin while the number of receptors remained constant (Olefsky, 1976). There is some evidence

that serine/threonine phosphorylation on specific sites on the insulin receptor reduces tyrosine kinase activity. Agents which increase intracellular levels of cAMP such as adrenaline for instance, are particularly adept at down-regulation of the insulin receptor in such a manner. It is thought the phosphorylative agent is cAMP kinase (Haring *et al.*, 1986, Stadtmauer and Rosen, 1986).

6.1.4 Chapter objectives

Results from chapter 5 suggested that differences may exist in insulin and glucose tolerance of high and low wool strength animals. Since hyperinsulinaemia often results in down-regulation of the insulin receptor, this chapter assessed if simultaneous changes in insulin receptor binding kinetics had occurred in the high wool strength animals.

6.2 Methods

6.2.1 Sampling procedure

At the end of the 1990 trial (21 September) skin biopsies were removed from the subset rams before they were turned out to pasture. An area of mid-side skin was surgically prepared with a razor to remove as much wool as possible so that contamination of the sample with wool proteins was reduced. Pentobarbitone sodium was administered at a final concentration of 24 mg/kg live weight (Nembutal, 60 mg/ml, TechVet Lab Ltd, NA) by a veterinary surgeon. Ellipsoid shaped pieces of skin approximately 10 cm in length and a maximum diameter of 3 cm, were collected under general anaesthesia. The skin biopsy sample was immediately frozen in liquid nitrogen and stored at -70^{0}C.

6.2.2 Preparation of particulate membrane fractions

Skin samples were used to prepare particulate membrane fractions for assessment of insulin binding (adapted from Alexandrides *et al.*, 1989). Approximately 15-20 g of frozen tissue was crushed between four layers of aluminium foil with a hammer. Crushed tissue (8-12 g), was homogenized on ice in ice-cold isolation buffer (5 ml/g tissue, 50 mM HEPES, 150 mM NaCl, 1 mM PMSF, with 1 TIU aprotinin/ml and bacitracin 100 units/ml adjusted to pH 7.6 with 10 M NaOH), for three 30 s bursts using an Ultraturrex homogenizer set at half speed. Cellular debris was pelleted by spinning at 9500 g for 10 minutes in a Sorvall centrifuge at 4 ^{0}C using the Ti70 rotor. Consequently, the supernatant containing the membrane fraction was centrifuged at 200 000 g for 45 minutes at 4 ^{0}C. The pellet was washed twice by resuspension in an equivalent amount of ice-cold isolation buffer and centrifugation as before. The washed pellets were resuspended in 7.5 ml of ice-cold isolation buffer. 50 µl aliquots of the suspension were assayed for protein in duplicate, according to the method of Lowry (1970) and employing a 0-2000 µg/ml range of BSA standards. The membrane suspensions were frozen in liquid nitrogen and stored at -70^{0}C until required.

6.2.3 Competitive binding assays

To assess insulin binding, competitive binding assays were prepared on ice in duplicate. Each tube contained an aliquot of membrane equivalent to 200 µg protein, 0.009 µCi ^{125}I-insulin, 50 µl of varying concentrations of cold competitor insulin and binding buffer to make up the final volume of 300 µl (100 mM HEPES, 120 mM NaCl, 600 nM $MgSO_4$, 9 mM glucose, 5 mM KCl, 1% BSA). Cold competitor ovine insulin was prepared in binding buffer to give a wide physiological range with final concentrations of 10E-06, 10E-07, 6.66E-08, 3.33E-08, E-08, 6.66E-09, 3.33E-09, E-09, 6.66E-10, 3.33E-10, E-10 M. Assays were incubated at 4 ^{0}C for 16 hours and reactions stopped by the addition of 400 µl ice-cold binding buffer. Membranes were pelleted at by centrifugation at 12 000 rpm at 4 ^{0}C for 20 minutes in a Heraeus microfuge. The supernatant was removed by aspiration and the pellets washed in a second 400 µl of binding buffer followed by a brief vortex. Centrifugation and removal of supernatant was repeated as before. The bottom portions of the Eppendorf tube containing the pellet were removed and transferred to polypropylene Wassermann tubes for counting. Assays were counted on an LKB Compu gamma counter for 10 minutes/tube.

6.2.4 Scatchard analysis

6.2.4.1 Interpretation of curvilinear plots

Insulin receptor binding data was interpreted by expressing the data as Scatchard plots. These plots are often curvilinear which has been interpreted as evidence of negative cooperativity between receptors or the presence of receptor populations of different affinity. Those in favour of a negative cooperativity model propose a decrease in binding affinity as occupancy of binding sites increase. This may results from dissociation of a high affinity $\alpha_2\beta_2$ tetramer into a low affinity $\alpha\beta$ dimer (De Meyts and Roth, 1975, De Meyts *et al.*, 1976). The biological role for negative cooperativity is thought to be an increase in insulin sensitivity at low concentrations of insulin due to preferential binding to high affinity receptors. As insulin concentrations increase, receptors move into a low affinity state which reduces sensitivity (Gliemann *et al.*, 1975).

A strong case for receptor heterogeneity in producing curvilinearity is provided by the evidence of different insulin receptor isoforms exhibiting different binding activity (see

section 6.1.1). Gammeltoft (1984) describes other objections to the negative cooperativity model which includes a lack of evidence for increased receptor dissociation as receptor occupancy increases. The most commonly used multiple binding site model was developed by Kahn *et al.* (1974). It suggests the existence of three binding sites: a high affinity, low affinity and non-specific sites. Other work which has employed this type of model for insulin receptor binding has been described by Francis (1990) and Willis (1990).

Exactly which mechanism decides curvilinearity of the Scatchard plot remains a controversial issue. Evidence for the negative cooperativity model is collectively summarised by Gammeltoft (1984) and Taylor *et al.* (1991). De Meyts and Roth (1975) described how binding data used for Scatchard analysis could not distinguish between multiple classes of independent sites and negative cooperativity. Although it is accepted that negative cooperativity may occur in ruminant models, in this work the Scatchard plot was interpreted according to the model described by Kahn *et al.* (1974) where receptor populations of different affinity are presumed to coexist. This model was chosen over one of negative cooperativity because of its previous application to sheep (Francis, 1990, Willis, 1990). Consequently, it allowed useful comparisons to be drawn.

6.2.4.2 Description of the Scatchard plot

The binding of insulin to its receptor is a reversible reaction similar to the process described by Scatchard (1949). Binding of the ligand is assessed with a competitive binding assay where binding of constant amounts of ^{125}I-insulin is assessed in the presence of a range of cold insulin concentrations. The quantities **boverf** (specifically bound/free ligand) and **btotM** (total insulin specifically bound, M) are then analyzed according to a two-site model. Scatchard (1949), while studying the kinetics of ion binding to proteins, developed the principles described by the following equation (from Kahn *et al.*, 1974), where [H] is the concentration of free hormone, [R] is the concentration of free receptor, and [HR] the concentration of hormone receptor complex. K (the slope) denotes the equilibrium or affinity constant and R^0 (the intercept or constant) denotes the total receptor population.

$$\frac{[HR]}{[H]} = K([R^0] - [HR])$$

It is now generally accepted that in the analysis of insulin and its receptor, the plot of

Scatchard plots fail to give a linear equation as the above equation suggests. Instead, insulin binding has been interpreted as occurring at three different types of sites: A high affinity site, a low affinity site and a nonspecific site. The Scatchard equation therefore becomes:

$$[HR] = K_1 [H] ([R_1^0] - [HR_1]) + K_2 [H] ([R_2^0] - [HR_2]) + K_3 [H]$$

By subtracting the nonspecific binding from each value of bound hormone the final Scatchard plot is described by

$$\frac{[HR] - K_3 [H]}{[H]} = K_1 ([R_1^0] - [HR_1]) + K_2 ([R_2^0] - [HR_2])$$

The equations are based on several assumptions (Kahn *et al.*, 1974):

i) The hormone is present in a homogenous form

ii) Labelled and unlabelled hormone behave identically

iii) Full equilibrium is achieved

iv) Bound and free hormone can be separated without disturbing the equilibrium

v) No cooperative effects exist between binding sites

vi) Hormone is univalent: one hormone molecule can react with one binding site.

Zierler (1989) discusses the correct way to resolve Scatchard plots. Klotz (1982) further discusses the problems which can be encountered with Scatchard analysis if certain precautions, such as a data set which covers the appropriate range of competitor insulin, are not taken. A further consideration is that the original curve must be regenerated by the addition of the two lines into which it was resolved (Zierler, 1989). Scatchard plots cannot be resolved by eye as has been done in the past (Feldman, 1983, Zierler, 1989). Instead, a clear mathematical model and computer analysis must be used to provide the

objectivity required of the analysis.

In this work, lines were fitted using the maxlik procedure of GAUSS 386 (appendix VII) which employs the quasi - Newton method described by Broyden (1965). The procedure minimized the least squares of the experimental data as it is plotted as bound/free versus total bound (M)/200 µg membrane protein. It resolves the resultant curvilinear plot in terms of two straight lines which when added along the radians yield the original curvilinear plot (Gliemann et al, 1985). The steep line (high affinity receptor population) and the flat lines (low affinity receptor population) provide information about receptor capacity and affinity by the magnitude of the x-axis intercept and the slope of the line respectively.

The data required as input for the Gauss program (bound/free and total bound (M)) were calculated at each cold competitor insulin concentration using the statistical package SAS (appendix VI). Following is a brief outline of the rationale used in the calculations. Steps 1-4 describe how the proportion of tracer bound at each concentration of cold competitor insulin is used to calculate bound over free. Steps 5-7 explain how the concentration of insulin bound (hot plus cold fractions) was calculated as a molarity per 200 ug of membrane protein.

1. The hot fraction bound (**tracer**) at the varying concentration of cold insulin was calculated by dividing the cpm at the various cold concentrations of cold competitor insulin (**coldcpm**) by the total counts added (**hotcpm**).

tracer = coldcpm/hotcpm eqn 1

2. This hot fraction bound (**tracer**) was adjusted for non-specific binding by multiplication by a correction factor (**1 - nsbind**). This correction factor allows for the nonspecific binding of insulin as described by Kahn *et al.* (1974). Non-specific binding was established as the fraction of total cpm bound when all the specific binding sites are occupied by a large saturating concentration of cold insulin (10E-06 M). The correction factor was calculated for each animal as follows.

spbind = tracer * (1 - nsbind) eqn 2

3. The fraction of free insulin was calculated next as one minus the hot fraction bound (which is the sum of specific and nonspecific binding, see eqn 1).

free = 1 - tracer eqn 3

4. Bound over free was calculated as the fraction specifically bound over the fraction free.

boverf = spbind/free eqn 4

5. The x-axis of the Scatchard curve represents the molarity of total insulin which is the sum of tracer and competitor insulin binding. While calculating this quantity it is assumed that the concentration of hot insulin available for binding is negligable when compared to the large amounts of cold insulin available. The relative contribution of hot insulin to total receptor binding is therefore minimal. Consequently, the molarity of the tracer specifically bound was only calculated once for each animal at a cold competitor insulin concentration of 10E-06. This figure was calculated for an equivalent of 200 ug of membrane fraction protein.

bhotM = (2.25E-16 (= moles of tracer in 0.1 mls)/reaction volume) * hotcpm * (1 - nsbind) eqn 5

6. Next the molarity of the cold insulin bound was calculated.

bcoldM = coldconc * spbind eqn 6

7. And finally the total M of insulin bound could be derived as the sum of the cold (eqn 6) and hot (eqn 5) insulin bound.

btotM = bhotM + bcoldM eqn 7

6.2.5 Klotz analysis

Scatchard analysis is prone to misinterpretation of receptor capacity, particularly if more than one receptor site is present (Klotz, 1982). Feldman (1983) in his discussion of the topic explains that this is because theoretically the binding curve of Scatchard coordinates should descend towards a intercept on the x-axis representing total receptor capacity. Experimental data however, never quite reaches this axis because the reversibility of the reaction makes sure that true saturation is never reached. Unfortunately, because of the nature of the Scatchard curve it may appear that it is quite valid to derive receptor capacity by extrapolating to the x-axis since the points have come so close. Klotz (1981) states that to give in to such temptation can grossly underestimate receptor capacity. He

recommends preparation of a Klotz curve instead (Bound (M) versus log free). Such a graph produces an S-shaped curve with an inflection point at half maximal binding and a plateau as the concentration of free ligand approaches very large infinite values (saturation). The value of bound ligand at which the plateau is reached presumably represents receptor capacity. He recommends that if one pursues with Scatchard analysis the data is checked on a Klotz curve in order to establish that the data has reached beyond the inflection point. If it does not, he reasons the data is not suitable for the calculation of receptor capacity and a wider range of cold competitor insulin should be used. To assess how close receptors came to saturation in this experiment, Klotz curves were prepared for two representative animals. The data plotted (Bound versus log Free) was provided by the quantity btotM (equation 7) calculated by the SAS program in appendix, while the quantity free was taken as the concentration of cold competitor insulin. Although it is accepted that the latter quantity will not be an accurate representation of free insulin given that some of it will have been bound by the particulate membrane fraction, this was not seen as significant for the purpose of this analysis.

6.3 Results

Particulate membrane fractions prepared from skin biopsies taken from the subset rams (n=12) were assessed for insulin binding (Figure 6.1). The suitability of the cold (competitive) insulin concentrations used was assessed according to the suggestions of Klotz (1981, see section 6.2.5, Figures 6.3 and 6.4) with the aim of evaluating how close receptor preparations had come to saturation (Figures 6.2 and 6.3). The curves suggest that the data points reached close to if not above an inflection point and it was therefore assumed that the experimental data was complete enough for the calculation of receptor capacity. Consequently, competitive binding data was resolved by plotting the data as outlined by Scatchard (1949) which describes the resultant curve by two lines according to a two-site model (Figures 6.4 and 6.5 and Appendix VIII). 'Steep' and 'flat' lines were produced with slopes and x-intercepts representing affinity and capacity of the high and low affinity insulin receptor populations respectively (Table 6.1). It was not possible to fit a flat line in four cases. This was because the line of best fit was horizontal and therefore of zero slope with an x-intercept nearing infinity (see figure 6.5). Subsequent correlations between low affinity insulin receptor binding and wool characteristics, were therefore carried out on a sample reduced to eight (Table 6.2).

Converting the capacity figures to M/mg membrane protein resulted in the average binding affinity and capacity for the low affinity receptor population being 3.0×10^{6} and 7.5 x 10-*8* M/mg membrane protein. The high affinity receptor population had an average affinity and capacity of 1.8×10^{9} and 2.5×10^{-10} M/mg membrane protein. The affinity constants for the high affinity receptor population were so variable that an average was only taken for those animals with a constant less than 63 (n=8).

No significant correlations were obtained between any parameter of insulin receptor binding and fleece quality (Table 6.2). There was a trend however, for lower receptor affinity and capacity for the low affinity population in high wool strength animals.

Tag	Flat Slope (E+05)	Flat X Int (E-08 M)	Steep Slope (E+09)	Steep X Int (E-11 M)	Residual SS Model	SSBV	SS	MINFD
209	-6.7	3.5	-840.0	2.5	0.19	-3.76	47.7	30.2
415	-10.7	1.8	-1.5	7.3	0.26	9.13	90.4	31.6
439	-23.7	0.8	-1300.0	3.3	0.22	-9.39	46.5	31.3
440	-8.9	2.6	-1.7	7.0	0.07	-10.22	52.8	31.0
448	-11.3	1.2	-2.1	5.2	0.03	-5.64	67.0	31.4
604	-110.0	0.3	-13000.0	1.8	0.29	6.22	92.3	33.4
617	-34.5	0.8	-2.5	4.9	0.18	5.56	74.7	28.2
618	Could not be fitted		-2.0	5.4	0.35	-3.34	93.1	32.4
621			-1.1	8.4	0.06	-1.95	88.9	35.6
623	-37.4	0.9	-2.2	4.6	0.09	4.56	65.2	30.9
640	Could not be fitted		-1.2	6.8	0.31	0.02	71.6	32.8
641			-63.0	2.2	0.43	14.24	98.7	36.5
Mean	-30.4	1.5	-1268.0	4.9	0.21	0.45	74.0	32.1
SEM	12.1	0.4	1073.6	0.6	0.04	2.18	5.4	0.7

Table 6.1: Insulin receptor binding data obtained from Scatchard plots

Shown are the slope and x intercepts (x int) for the flat and steep lines used to resolve a curvilinear Scatchard plot of Bound insulin/Free insulin versus the total insulin bound (M). Capacity (x intercepts) is presented as M/200 ug protein. The residual sum of squares after the resolution of the curvilinear plot by a program written for the Gauss package are also given (Appendix VI). The table also included the raw fleece data used for the correlative analysis of Table 6.2.

	SSBV	SS	minFD
Flat Slope n = 8	-0.46	-0.58	-0.51
Flat X Int n = 8	-0.38	-0.50	-0.21
Steep Slope n = 12	-0.19	-0.24	-0.15
Steep X Int n = 12	-0.25	0.09	0.01

Table 6.2: Association between parameters of fleece quality and insulin receptor binding. Shown are Pearson correlation coefficients. None of the correlations were significant ($p > 0.10$).

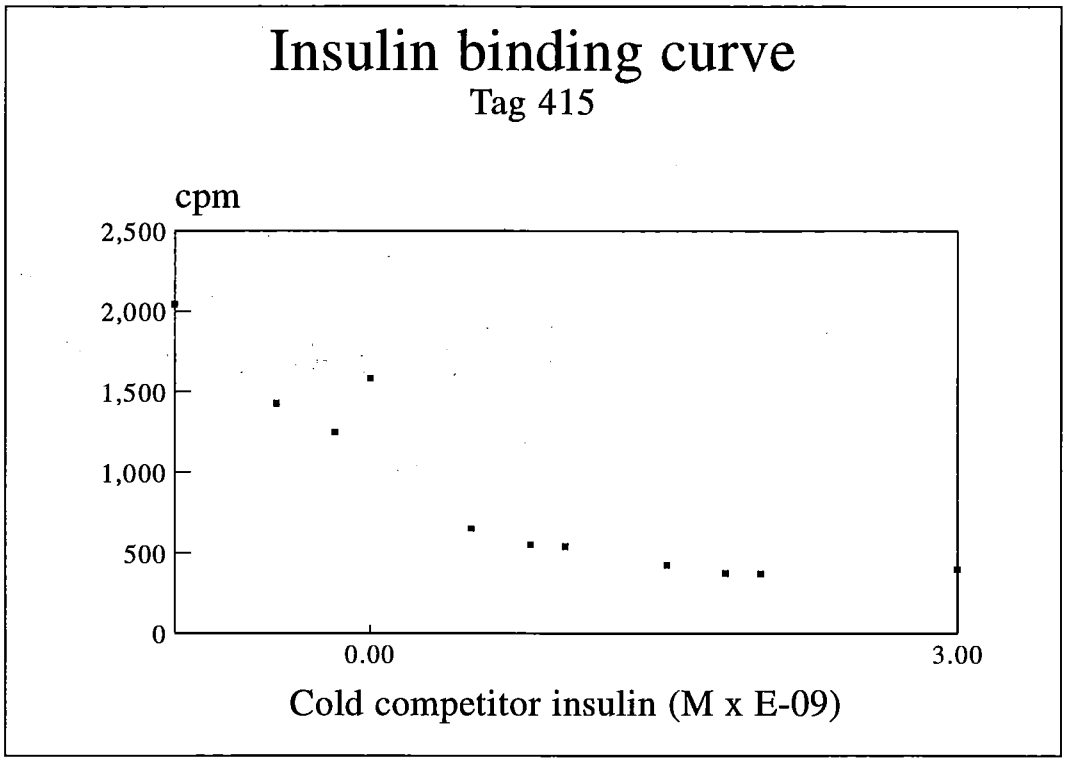


Figure 6.1: Results from a competitive binding assay

Binding is assessed as the radioactivity of the pellet in response to varying concentrations of 'cold' competitor insulin. These data are subsequently used for Scatchard analysis.

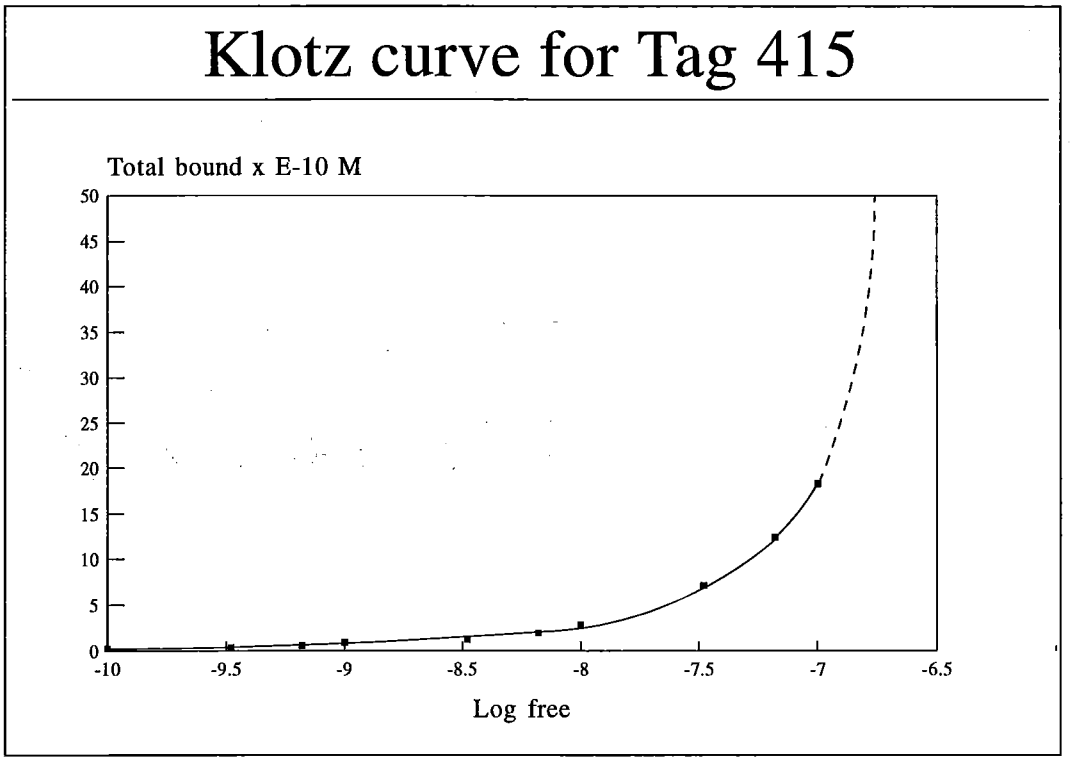


Figure 6.2: Klotz curve for Tag 415

Insulin receptor binding data presented as suggested and explained by Klotz (1981, see section 6.2.5). If the cold competitor insulin concentrations used in the binding assay were high enough to achieve close-to-saturation insulin receptor binding, then obtaining an estimate for receptor capacity from a Scatchard plot is justified. This can be assessed from the S-shaped 'Klotz' curve with an inflection point at half-maximal binding and a plateau when full saturation is reached. Clearly, for this animal, a greater range of cold competitor insulin should have been used.

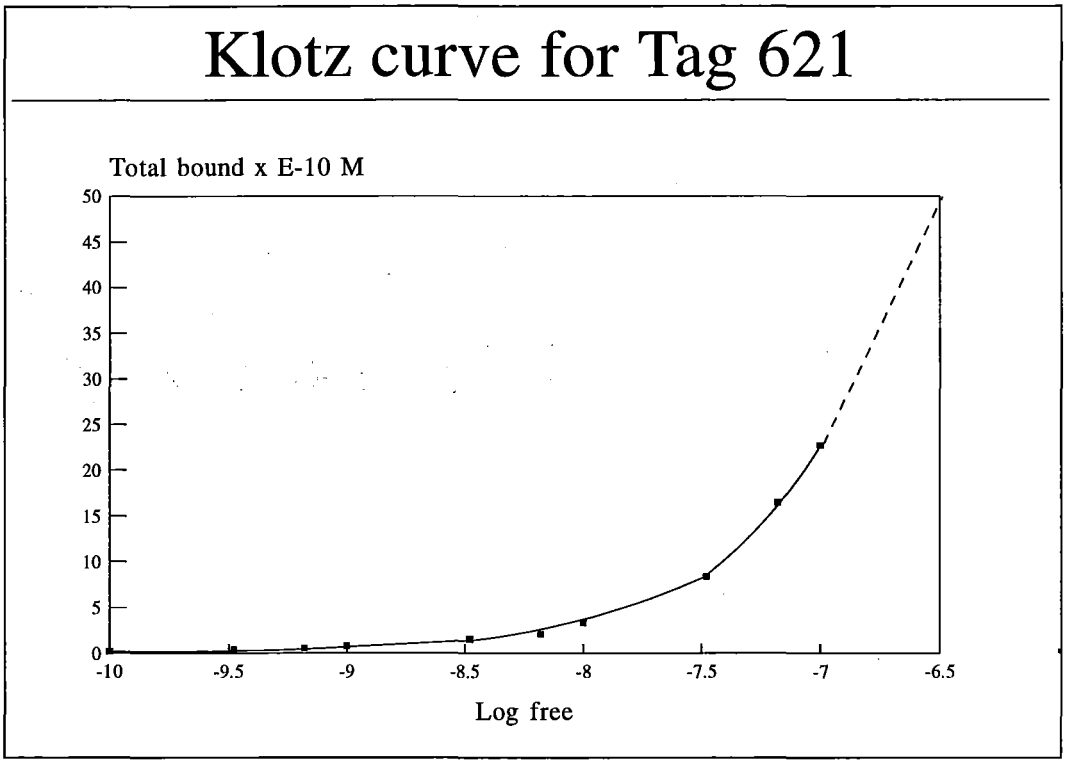


Figure 6.3: Klotz curve for Tag 621

The data set for this animal is more suitable for Scatchard analysis than that for animal 415 (see Figure 6.2). The curve appears to have come close to an inflection point and gives a better estimation of the concentration at which receptor saturation occurs.

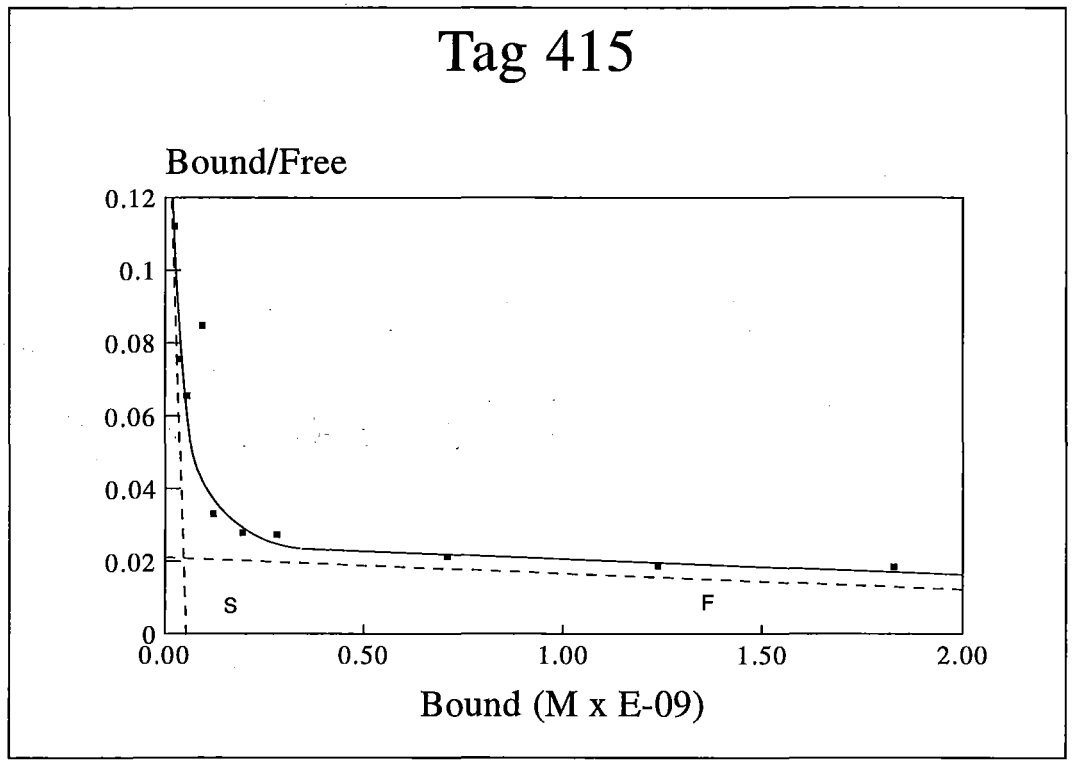


Figure 6.4: Scatchard plot for tag 415

The curvilinear plot of bound:free hormone versus bound hormone has been resolved into two lines (dotted) by a computer program (see section 6.2.4). The steep line (S) represents binding to the high affinity receptor population while the flat line (F) represents binding to the low affinity receptor population. The slope of each line equates to receptor affinity while the x-intercept equates to receptor capacity.

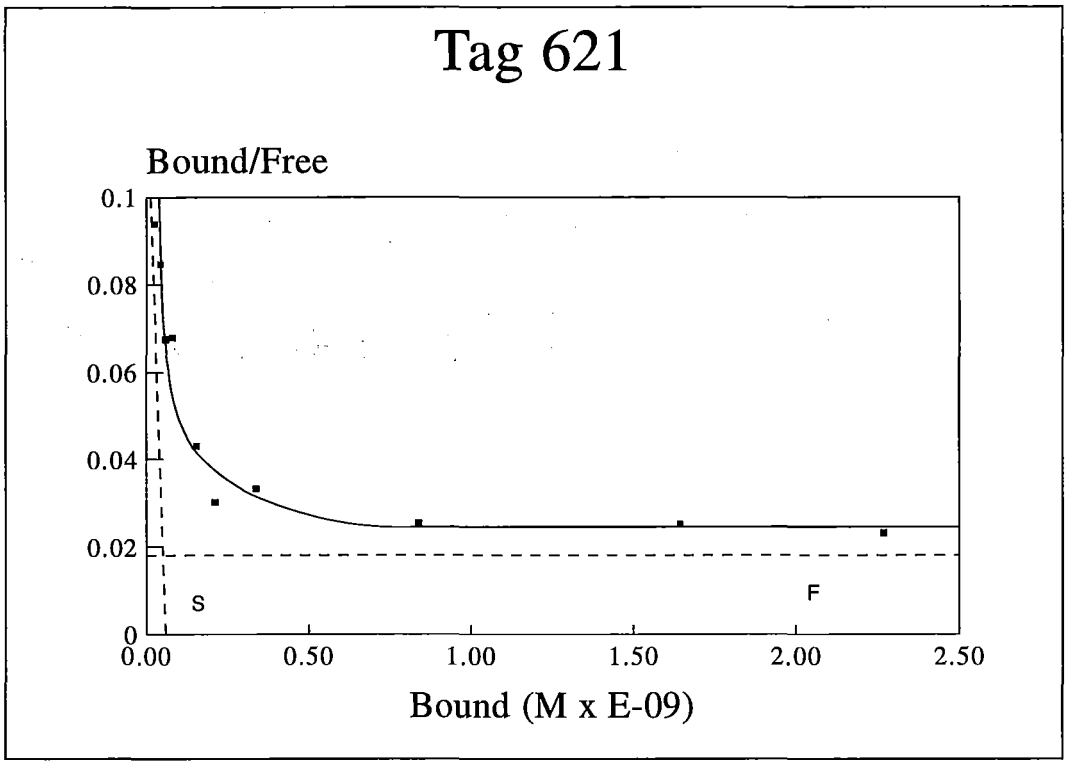


Figure 6.5: Scatchard plot for which the flat line (F) did not yield a slope or x-interecept.

6.4 Discussion

6.4.1 Suitability of a two-site model in this study

The data from competitive insulin binding assays used in Scatchard analysis was moderately variable between replicates and highly variable between animals. Grigorescu (1988) suggests that in computer-assisted statistical Scatchard curve fitting as used in this work, the sum of squares is a major criterion for measuring the goodness of fit of the model. In this study, the residual SS varied from close to zero (0.028) to 0.429 (table 6.1). This suggests, that while the model adequately fitted some of the data, it fitted the data from other animals poorly. Visual assessment of the raw data suggested a reasonable amount of variability occurred between replicates. It would be wise therefore to increase the number of replicates in future experiments. However, by far the largest amount of variability occurred between animals (see table 6.1).

Insulin receptor binding was assumed to occur at three sites as described by Kahn *et al.* (1974): i.e. a high affinity, a low affinity, and a nonspecific binding site. Consequently, the curvilinear Scatchard plots produced in this work were resolved into steep (S) and flat (F) lines representing the high and low affinity receptor populations, and a non-specific fraction (the third binding site). In four instances the F line was of horizontal or negative slope. This provided meaningless data, since it meant that theoretically, the low affinity receptor was present in infinitely large concentration or non-existent. Furthermore, the affinity of the receptor was either zero or repellent for its ligand being of zero or positive slope. The most sensible interpretation of the data is that these animals lacked low affinity insulin receptor binding sites. In support, in Kahn *et al.* (1974), seven investigators reported heterogeneity of binding sites (i.e. more than one insulin receptor type), while three investigators reported homogeneity and the biological significance of low affinity receptor site has been questioned by Cuatrecasas (1972) and Marinetti *et al.* (1972). In addition, Francis (1990) when studying ovine adipose tissue, was not able to resolve a Scatchard plot into two lines for five out of twelve animals. She chose to analyze the binding data by resolving the Scatchard plot into one line to represent a single high affinity receptor population. This work is therefore not the only study in which a low affinity receptor population has apparently not been found.

An alternative explanation for the apparent lack of low affinity receptor sites for some animals, may be a technique related phenomenon. Scatchard (1949) pointed out that if

analysis depends on the value of the intercepts, one must be certain that the experimental points extend over a range that brings their extremities sufficiently close to the intercept to justify extrapolation. Certainly, the Klotz curves (Figures 6.2 and 6.3) suggest that although the data points may have come close to the curve's inflection point for some animals, more data points at higher concentrations of cold competitor insulin would have been desirable. Unfortunately animal ethics considerations prevented the sampling from being repeated (there was not enough material from the previous sampling for a repeat experiment). Only few points define the shape of the horizontal sector of the Scatchard curve. It is therefore easy to see how lines with negative or horizontal slope could occur for the low affinity receptor population. Interestingly, there was a trend for the Scatchard curves to slope up at the point of greatest total binding (see figure 6.4 and appendix VIII). This trend may also have reduced the fit of the model.

6.4.2 Comparison of binding kinetics with those of others

It is not known why the between animal variability in receptor binding kinetics was large, particularly with regard to receptor capacity, but this problem also hindered the interpretation of the work of Francis (1990) and Willis (1990). This raises some concern over the suitability of the technique since all these studies were performed in the same laboratory. Future studies should reassess the suitability of this technique.

Comparison of capacity data with the work of others is made difficult by the large number of ways that the experimental data can be expressed and the variety of different experimental conditions employed. The latter poses a problem because insulin receptor binding is highly dependent on temperature and pH. Consequently, results could only be compared with those who expressed capacity in terms of M/mg of membrane protein and studied binding at 4^0C and a pH of 7.6, conditions chosen because they reduce receptor degradation.

Sheep skin appears to have significantly more insulin receptors than rat liver (Kahn *et al.*, 1974) and the affinity of the sheep skin receptor compared to rat liver is equal for the high receptor population but significantly less for the low receptor population. The high affinity receptor population had an affinity constant of 3.3×10^9 and a capacity of 2.1×10^{-13} M/mg membrane protein and the low affinity receptor population had an affinity constant of 4.5×10^8 and a capacity of 3.1×10^{-12} M/mg membrane protein. In rat liver, the low affinity receptor population therefore exhibited a 7-fold reduction in affinity and an approximate 15-fold increase in capacity. Results presented here showed the high

affinity receptor population to have an average affinity constant of approximately 2×10^9 and a capacity of 2.5×10^{-10} moles per mg membrane protein. The low affinity receptor population had an average affinity constant of 3×10^6 and a capacity of 7.5×10^{-8} M/mg of membrane protein. The low affinity receptor therefore exhibited a 700-fold reduction in affinity and a 300-fold increase in capacity over the high affinity receptor. The relative differences in affinity and capacity between the high and low affinity receptors between rat liver and sheep skin was therefore significantly different.

Affinity constants for other tissues have been previously shown to vary from 2E10 to 1.3E05 (Kahn *et al.*, 1974). The majority are of an affinity around 2×10^8. This study showed that the capacity of both receptor populations in skin was similar if not increased over the capacity of ovine fat and muscle tissue (Willis, 1990). Ovine tissues may therefore have a greater capacity to bind insulin than those of the rat, specifically rat liver (Kahn *et al.*, 1974).

6.4.3 Biological significance of results

Potential differences in the partitioning of nutrients in favour of the wool follicle could arise from differences in sensitivity and capacity of insulin receptor binding. However, no significant correlations between insulin receptor binding and wool strength were observed. There was a small trend for a decrease in low affinity receptor capacity and affinity in the high wool strength animals (Table 6.2).

Hyperinsulinaemia leads to insulin resistance in man (Gavin *et al.*, 1974). This may occur via a decrease in the number of receptors on the cell surface (down-regulation). Chapter 5 produced evidence that the high wool strength animals had greater plasma insulin and were insulin resistant. The small decrease in low affinity receptor capacity observed may therefore be a form of down-regulation from the elevated levels of plasma insulin in high wool strength animals. Decreases in capacity result in a rightward shift in the dose-response curve for insulin activity (Kahn, 1978). This has the effect of decreasing the sensitivity to insulin while the ability of the cell to respond to insulin remains the same. However, the large amount of spare receptors shown in several systems (the spare receptor theory, see section 6.1.2), means that the responsiveness of the cell may not be reduced until only 10-30% of the receptors remain (depending on cell type). It is therefore not known if a reduction in receptor capacity could potentially change the responsiveness of sheep skin to insulin.

The lack of significant correlations between binding data and wool strength (the latter being the result of wool growth) means that it is unlikely that differences in insulin receptor binding between animals is a significant factor in producing the observed differences in wool strength. This does not rule out that post receptor events (discussed in section 7.1), including insulin receptor tyrosine kinase activity, may produce changes in wool strength.

Candidate gene expression in the wool follicle

7.1 Introduction

Insulin has been shown to regulate a plethora of genes at both transcriptional and translational levels. Many of the gene products direct the synthesis of enzymes which have a well established connection to glucose metabolism, while others represent major secretory proteins/hormones, integral membrane proteins and oncogenes/transcription factors (Table 7.1). As discussed in chapters 5 and 6, staple strength may be associated with changes in gene expression within the wool follicle. Given that physiological data of Chapter 5 has eluded to a difference in glucose uptake and plasma insulin levels between high and low wool strength animals, a number of genes regulated by insulin make good potential candidates for further study. Potentially, regulation of glucose uptake via transcriptional modulation of these key loci could affect the rate of keratin synthesis and cell division, thereby modulating fibre cell size, number, and consequently fibre diameter.

7.1.1 Selection of probes

In this work, only a limited number of candidate genes of the many potentially involved in regulating wool growth could be studied. A brief description of the selected genes follows.

7.1.1.1 Insulin Receptor

Insulin has been shown to be a potent growth factor (Koontz and Iwahashi, 1981, Moses and Tsuzaki, 1991), stimulating both nutrient uptake and cell division (via DNA synthesis). For insulin's stimulatory effects to be realised, it has to bind to an insulin receptor on the cell surface. The extent of insulin stimulated activity within a cell is

Table 7.1: Regulation of gene expression by insulin (adapted from O'Brien and Granner, 1991)

Intracellular enzymes	**Effect on transcription**
Pyruvate kinase	+
ATP citrate lyase	+
Serine dehydratase	-
Fatty acid synthtase	+
Glutamine synthetase	+/-
Ornithine decarboxylase	+
Tyrosine amintransferase	+/-
Aspartate amintransferase	-
Fructose-1,6-bisphosphatase	-
Protein disulphide isomerase	-
Carbamoyl-phosphatase synthetase I	-
Phosphoenolpyruvate carboxykinase	-
Glucose-6-phosphate dehydrogenase	+
Glycerol-3-phosphate dehydrogenase	+
Brown adipose tissue uncoupling protein	+
Glyceraldehyde-3-phosphate dehydrogenase	+
6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase	+
Malic enzyme	+
Glucokinase	+
Aldolase B	+
Integral membrane proteins	
Insulin receptor	+/-
Growth hormone receptor	+
GLUT1	+
GLUT2	+
GLUT4	+
Proteins involved in reproduction	
Casein	+
Ovalbumin	+
Secreted proteins/hormones	
IGF 1	+
IGF 2	+/-
Prolactin	+
Glucagon	-
Growth hormone	-
Apolipoprotein B	-
Lipoprotein lipase	+
Hepatitis B surface antigen	-
Pulmonary surfactant apolipoprotein A	-
IGF binding protein 1	-
IGF binding protein 2	-
α-2_u globulin	+
α-Amylase	+
Albumin	+/-

Adipsin	+/-
Transcription factors	
c-fos	+
egr-1	+
c-jun, Jun B, Jun D	+
c-myc	+
Miscellaneous	
Gene 33	+
Thyroglobulin	+
Proline-rich protein	+
Quiescence-specific gene	-
τ-Crystallin	+
hsp 70	+
S14	+
c-Ha-*ras*	+

therefore a function of external concentrations of insulin as well as the number and affinity of binding sites available. This can be aptly demonstrated by certain breast tumours which show an increase in insulin receptors (Frittitta *et al.*, 1993) which presumably causes the tumour by a marked increase in the mitogenic potential of insulin. Conversely, in disorders such as leprechaunism, levels of insulin receptor mRNA are greatly reduced (Moses and Tsuzaki, 1991). Insulin receptor mRNA levels are also modulated as part of 'normal' metabolism. Receptor mRNA is down-regulated in response to insulin (Okabayashi *et al.*, 1989) and increased during muscle differentiation (Mamula *et al.*, 1990). Hormones such as glucocorticoids can also cause a 5-10 fold increase in insulin receptor mRNA (Mamula *et al.*, 1990). Since the insulin receptor binding data of chapter 6 was too variable to be conclusive, a study of its mRNA could clarify the role of the insulin receptor in promoting wool growth.

Because of the different ways the insulin receptor gene transcript can be processed to mRNA (Anderson *et al.*, 1993, Norgren *et al.*, 1993, see section 6.1.1), mRNAs of several sizes have been observed in human tissues (Ullrich *et al.*, 1985, Taylor *et al.*, 1991).

7.1.1.2 IGF-I Receptor

The structural similarity between the insulin and IGF-1 receptors is such that they can be bound by the same monoclonal antibody (Roth *et al.*, 1983). In addition, the IGF-1 receptor can bind insulin as well as IGF-1 albeit at 100-500 fold lower affinity (Werner *et al.*, 1993). Some workers have suggested the mitogenic events related to insulin are mediated via the IGF-1 receptor in tissues traditionally not seen as insulin-responsive (e.g. fibroblasts), particularly at higher levels of plasma insulin (Moses and Tsusaki, 1991). Furthermore, blocking of the IGF-1 receptor with monoclonal antibodies abolishes the mitogenic effects of insulin on cultured human fibroblasts (Van Wyk *et al.*, 1985). There is some controversy surrounding this observation, since Flier *et al.* (1986) failed to reproduce the above results and mitogenic effects of insulin have been observed in cells without IGF-1 receptors (Koontz and Iwahashi, 1981). The possibility exists however, that at high plasma insulin levels (observed in the high wool strength animals) insulin exerts mitogenic effects via the IGF-1 receptor in skin. Importantly, both the binding of IGF-1 (Flier *et al.*, 1986) and insulin (Baldwin *et al.*, 1981) have been observed in human fibroblasts. Insulin could therefore promote wool follicle metabolism both by increasing nutrient uptake via the insulin receptor in addition to promoting cell division via the IGF-1 receptor.

Northern blots of human, rat and mouse tissues have revealed a single 11 kb band when hybridized to a human IGF-1 receptor cDNA (Ullrich *et al.*, 1986). IGF-1 receptor mRNA levels show marked changes in response to development (Schober *et al.*, 1990) and may increase in certain tissues of experimentally induced diabetic animals e.g. kidney (Werner *et al.*, 1990).

7.1.1.3 Facilitative glucose transporters

There have now been seven facilitative glucose transporters genes reported in the literature (Klip *et al.*, 1994). These are GLUT1 (erythrocyte type, but generally ubiquitous), GLUT2 (liver, small intestine and pancreas type), GLUT3 (brain type), GLUT4 (muscle/fat insulin-responsive type), GLUT5 (small intestine fructose transporter type), GLUT6 (a pseudogene, ubiquitous) and GLUT7 (liver - intracellular type). Although the dominant sites of expression of these glucose transporters is described as above, expression of most have also been reported in other tissues. Because of the biology surrounding these glucose transporters (refer to Kahn and Flier,1990, Kasanicki and Pilch, 1990, Mueckler, 1990, Elsas and Longo, 1992, Gould and Holman, 1993, Klip *et al.*, 1994), GLUT1 and GLUT4 loci appeared to be the most suitable for further study as candidate genes of wool

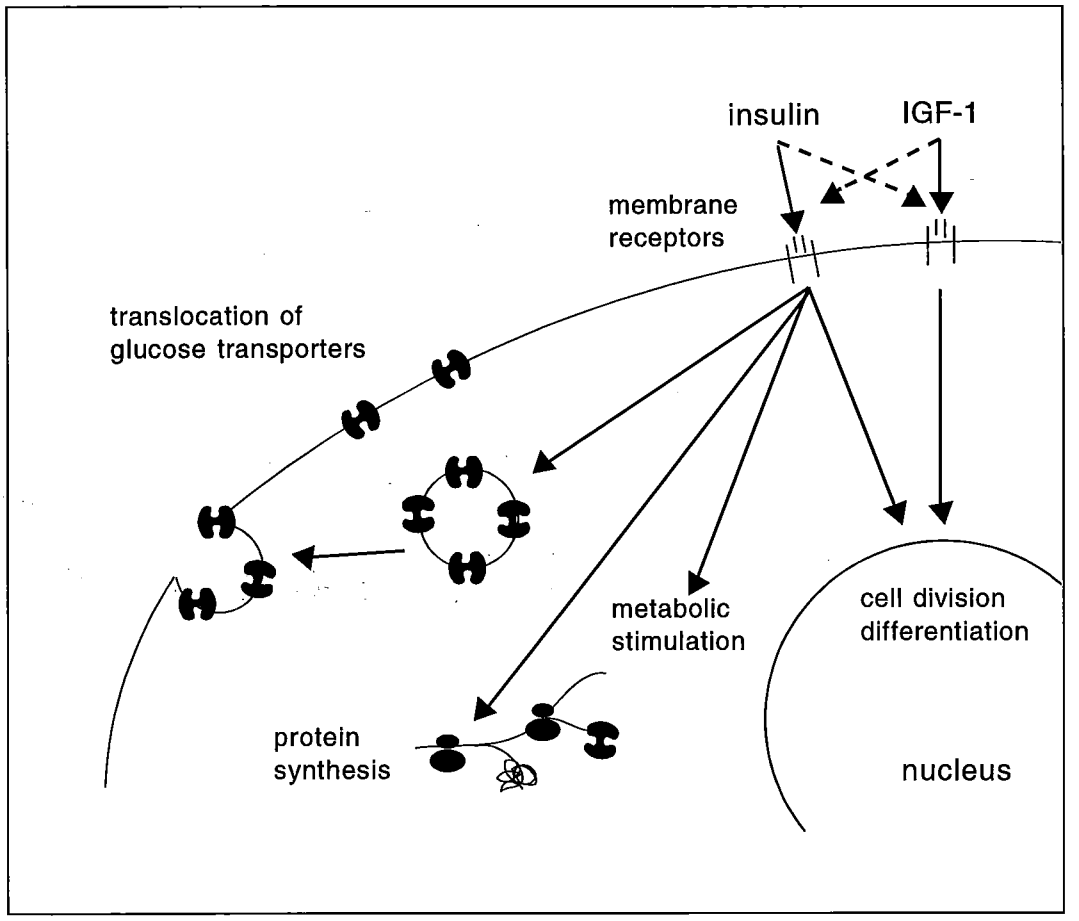


Figure 7.1: Theoretical basis of candidate gene selection

This figure gives a schematic representation of the hypothesis to be tested. Wool follicle cells display insulin (IR) and insulin like growth factor I (IGF-1R) receptors. The number of insulin and IGF-1 receptors on the cell surface are reflected by their mRNA levels within the cell (changes in receptor number may alter the responsiveness of the wool follicle to insulin in terms of its effects on glucose and amino acid uptake and DNA synthesis). Between animal differences in insulin stimulated glucose uptake can also occur via transcriptional activation of GLUT1 and GLUT4 expression. Consequently, these genes were also included in the study. Lastly, expression of the ribosomal protein S6 gene (S6) should be assessed because of its involvement in the initiation of translation and its regulation by insulin, albeit at a translational level. Differences in rpS6 mRNA levels could therefore reflect differences in the rate of initiation of protein synthesis.

strength.

The GLUT1 transporter is involved in basal glucose uptake and is localized at the plasma membrane in the basal state. Highest levels of GLUT1 mRNA are normally found in the brain where it is the principle transporter mediating the uptake of glucose across the blood-brain barrier (Dwyer and Pardridge, 1993). It is also enriched in the cells of the blood-tissue barriers such as the blood-brain barrier, the placenta and the retina (Froehner *et al.*, 1988). Increases in GLUT1 expression are often associated with a rise in metabolic activity. For example, Brown and Wahl (1993) indicated that GLUT1, not GLUT2 or GLUT4, is expressed highly in breast cancers. Furthermore, pregnancy and lactation cause a progressive increase in expression of GLUT1 in rat mammary gland (Camps *et al.*, 1994) suggesting that GLUT1 is involved in partitioning glucose between different tissues in response to certain stimuli. At the peak of lactation, GLUT1 protein levels were greater in the mammary gland than in other GLUT1 enriched tissues such as the brain. In contrast, GLUT4 decreased during pregnancy and practically disappeared during lactation.

GLUT4 is the major glucose transporter of muscle and adipose tissue, and is highly insulin responsive. In the basal state GLUT4 transporter is largely sequestered by intracellular organelles (Slot *et al.*, 1991). However, when tissues containing GLUT4 are exposed to insulin, GLUT4 is translocated to the cell surface to initiate glucose uptake (Slot *et al.*, 1991). Levels of this protein also respond markedly to exercise. Athletes, can adapt their metabolism by enhanced muscle blood flow and glucose uptake, which is in part achieved by an increase in GLUT4 protein (Ebeling *et al.*, 1993). Furthermore, while glucose uptake via GLUT4 into rat skeletal muscle decreased with age and obesity, it increased with chronic exercise (Ezaki *et al.*, 1992). Importantly, the level of skeletal muscle GLUT4 protein correlates highly with insulin-stimulated whole body glucose disposal in man (Koranyi *et al.*, 1991).

Insulin has been shown to regulate the abundance of both GLUT1 (Kosaki *et al.*, 1991, Klip *et al.*, 1994) and GLUT4 mRNA and protein (Yki-Jarvinen *et al.*, 1992, Flores-Riveros *et al.*, 1993, Klip *et al.*, 1994). However, generally speaking the abundance of GLUT1 in not affected by **acute** insulin treatment and only shows mild recruitment to the cell surface in response to insulin. **Chronic** insulin and glucose deprivation on the other hand result in a sustained increase of GLUT1 mRNA (Walker *et al.*, 1990, Kosaki *et al.*, 1991). Importantly, regulation of these two glucose transporters occurs via different mechanisms in fat and muscle which potentially could have a differential effect on how glucose is partitioned to either of these tissues in response to various stimuli. For instance, hyperglycaemia down-regulates levels of GLUT4 protein in muscle but not in fat whereas hyperglycaemia down-regulates GLUT4 mRNA in fat but not in muscle (Klip *et al.*, 1994). Furthermore, insulin is

a potent stimulant of GLUT4 transcription in fat but not in muscle (Postic *et al.*, 1993), a finding supported by Cusin *et al.*, (1990) who also suggested that this provided a mechanism for increased fat deposition and insulin resistance. Adipose tissue and muscle are not the only tissues showing differences in glucose transporter regulation. Observations of Kaiser *et al.* (1993) have indicated that smooth muscle cells can down-regulate GLUT1 protein concentration in response to hypoglycaemia but the aortic endothelial cells cannot. Cell culture experiments have also shown that hypoglycaemia caused a rapid increase in GLUT1 mRNA and protein. Hyperglycaemia on the other hand did not produce a decrease in GLUT1 mRNA and protein (Kosaki *et al.*, 1991).

Evidence is now accumulating which suggests that the ratio of GLUT1:GLUT4 may be an important correlant of physiological changes responsible for modulation of glucose uptake. This is suggested by an absence of tissues which only have the GLUT4 isoform and evidence such as that of Block *et al.*, (1991). This group has proposed that changes in GLUT1/GLUT4 ratios occur in muscle cells which have been either denervated, are becoming insulin resistant or are beginning to de-differentiate and that these cellular states tend to be associated with a greater proportion of GLUT1 protein.

Recruitment to the cell surface and stimulation of transcription may not be the sole regulatory modes of glucose transporter function. Glucose transporter activity may also be stimulated via conformational changes of the transporter (Karnieli *et al.*, 1993).

7.1.1.4 Ribosomal protein S6

Ribosomal protein S6 (rpS6) is found on the 40S subunit of the ribosome, and has been shown to be progressively phosphorylated on up to 5 tyrosine units in the presence of insulin-receptor kinase (Sugden and Fuller, 1991). This protein is thought to play a major role in the initiation of translation (Krieg *et al.*, 1988) and therefore has the potential to play a major part in the rate of keratin protein synthesis. It is thought that phosphorylation of rpS6 in response to insulin is mediated by MAP kinase via phosphorylation of S6 kinase (Conti, *et al., 1991*). S6 kinase has been implicated in mediating the mitogenic effects of insulin in studies performed by Hecht and Straus (1986).

Literature regarding the regulation of rpS6, particularly in terms of its mRNA trails behind that of other genes regulated by insulin such as the glucose transporters. Although no reports have been found regarding regulation of rpS6 transcription it is possible that

insulin regulates this gene both at a transcriptional and post-translational level. This has been found to occur for many other proteins regulated by insulin.

7.1.1.5 β - actin

B-actin is not regulated by insulin and was included in this study to provide a comparison for insulin-regulated candidate gene expression. It has traditionally been used in expression studies to normalise lanes for loading differences.

B-actin is an isoform found in tissues other than muscle and is thought to play an important role in providing the cytoskeleton and in cell division. While not regulated by insulin, it has recently been concluded that this gene can be acutely regulated by some other hormones such as dexamethasone (Marceau *et al.*, 1992). In addition, for actin expression to occur in cell culture, insulin is required in a permissive sense (Marceau *et al.*, 1992). Consequently, normalisation of lanes with respect to B-actin expression was used in conjunction with another method (see section 7.2.5.3).

7.1.2 The need for an isolated infusion model

While studying candidate gene expression between individuals it became apparent that expression of the GLUT1, GLUT4, IR and IGF-IR genes was extremely low or undetectable in ovine tissues. This was because autoradiographs had to be down for long periods of time (3-5 weeks) to achieve weak or no hybridization signal despite the 10-copy control giving strong signals. Furthermore, communication with other laboratories (Lobley, 1992, 1994, Bennet, 1994) has further confirmed that low expression is a problem for studies including glucose transporters in ruminant tissues. Consequently, the effect of an insulin infusion on candidate gene expression in an isolated skin patch was studied with the aim of: 1) establishing if this caused an induction in candidate gene expression 2) if the response to insulin was different between high and low wool strength animals. A technique first described by Hoey and Hopkins (1983) and further developed by Harris *et al.*, (1988, 1989) was used to catheterise the deep circumflex artery and vein supplying a defined patch of skin situated on the flank. Advantages of the technique are that infusion of the patch does not appear to affect metabolism at a systemic level. This allows the effect of insulin on the infused tissue to be separated from counter-regulatory mechanisms. In addition, an accurate measure of the insulin levels of the blood supplying the wool follicles involved in the study can be obtained. Disadvantages of the technique are that the catheterization is a difficult and lengthy procedure and some problems with the infused patch shifting during the course of the experiment have been encountered.

7.2 Methods

7.2.1 Animal sampling procedures

7.2.1.1 For DNA extraction

Rodent genomic DNA was extracted from liver after the animal was killed by asphyxiation.

Human and sheep genomic DNA was extracted from blood obtained by venipuncture and collected into heparinized Venoject tubes.

7.2.1.2 For RNA extraction

Wool follicles were obtained using epilation by embedding the protruding wool fibres in a fast curing resin (Dentimex, Medic Corporation, Vulcan Lane, Christchurch, N.Z.) developed for the making of dentures. A scanning electron micrograph is presented in Figure 7.2 displaying the morphology of the sample obtained in this manner. The area intended for sampling of skin and wool follicles was clipped closely (the mid-side). Dentimex was prepared according to manufacturers instructions and spread over a 5x5 cm area within the clipped region taking care to thoroughly soak the wool and skin. A Dentimex impregnated fibre glass strip (5x5 cm) was placed on top of the soaked area and pressed down firmly. The patch was allowed to harden (approximately 10 min on warm dry skin), and removed after slaughter or under general anaesthesia before placing it in liquid nitrogen. Where tissues other than wool follicles were collected, the procedure was as follows. Working quickly, the skin sample underneath the wool follicle patch (now devoid of wool follicles) and the skin sample on the closely clipped area adjacent to the patch (approximately 2.5 cm^2 - with wool follicles) were removed. Subcutaneous fat was taken from the midside and muscle from the *vastus lateralis* of the hindlimb. After acquiring a liver sample, access to the brain cavity was obtained with a hacksaw. Samples other than wool follicles were immediately stored in solution D (see appendix IX) and placed on ice.

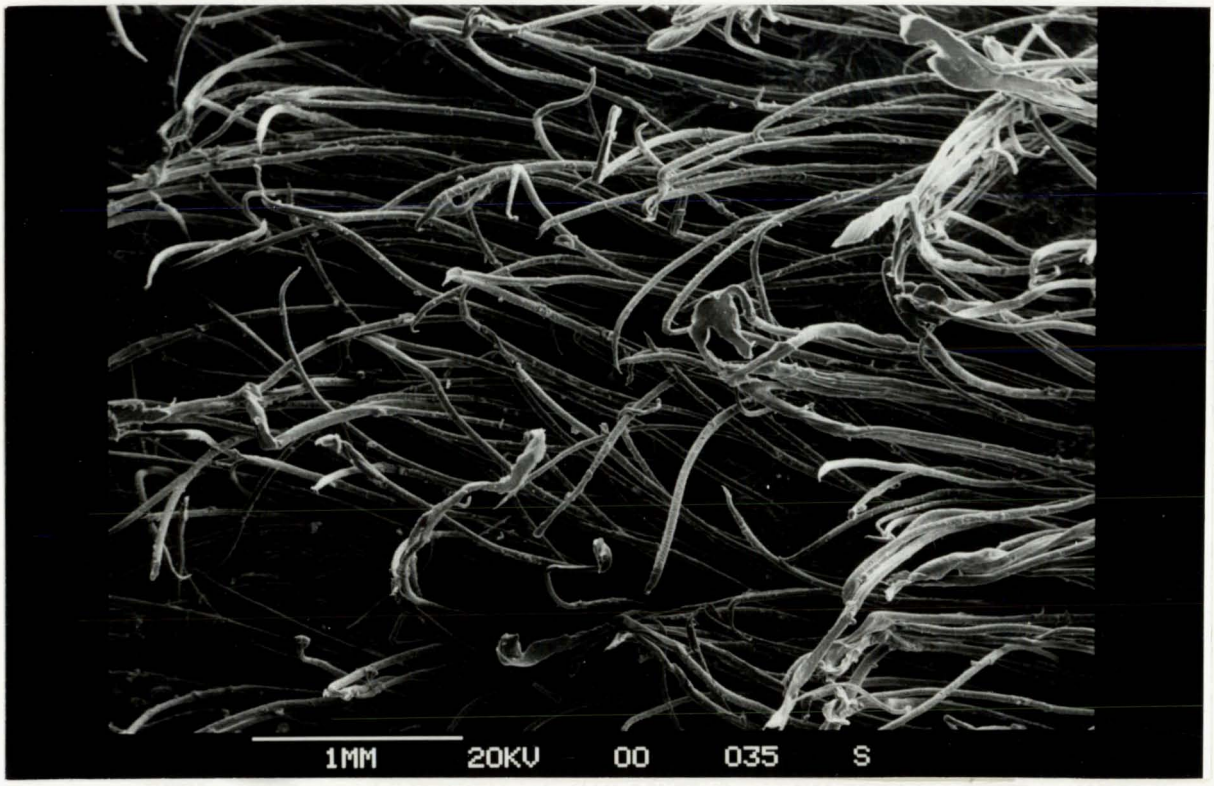


Figure 7.2: Scanning electron micrograph of epilated wool follicles used in total RNA extraction. Both primary and secondary follicles are visible with the follicle bulb clearly intact.

7.2.2 Infusion of insulin into an isolated skin patch

Prior to catheterization, 50 cm lengths of polyvinylchloride (PVC) tubing (internal diameter of 1 mm, external diameter of 1.2 mm) were prepared by marking a 10 cm sector into 1 cm segments with a permanent ink pen. These gradations allowed the depth of insertion of the catheter into the vessel to be determined (usually around 6 cm). Infusion lines consisting of equivalent PVC tubing were also prepared. Connections between lines were fashioned from blunt ended 19 gauge needles.

Catheterization proceeded as follows. The animals were anaesthetized using closed circuit anaesthetizing equipment and Fluothane (Halothane Ph. Eur. (ICI Pharmaceuticals, ICI Australia Operations Pty Ltd, Pharmaceuticals Division, 1 Nicholson Street, Victoria 3000). A 10 cm incision was made along the medial aspect of the femur and the skin was clamped aside to expose the working area. The deep circumflex iliac artery and vein were exposed, quickly catheterized in the region of the subiliac lymph gland and the vessels

were tied around the catheters using suturing cotton. A 45^0 cut on the end of the catheter aided insertion.

Patency was checked by assessing the ease at which venous blood could be removed, and by the successful infusion of Patent blue (a radio-opaque dye). Successful infusion of the dye allowed the area of infusion to be defined (approximately 20-30 cm^2) by a change in colour. If patency was inadequate the ties holding the catheters in place were loosened and the tubing inserted further or slightly retrieved. Once patency was established the wound was temporarily closed with michel clips.

The infusate consisted of 3800 μU insulin/ml sterile saline (0.09% sodium chloride) which contained 0.25% bovine serum albumin to stop insulin from adhering to the wall of the infusion line. Infusate was pumped into the infusion line using a peristaltic pump (Watson Marlow 302F, 12 lines). Care was taken to release the vacuum arising in the infusate bottle during infusion by inserting a needle capped by a sterile air filter into the rubber bung on the bottle. The rate of infusion was constant and amounted to 0.32 g/minute.

Sampling consisted of the collection of venous blood samples from the infused site and the jugular at 0 and 30 minutes and at 2 and 5 hours after the initiation of infusion. The jugular blood sample was taken for a base level insulin value control. Wool follicle samples were collected from a control shoulder patch and the infused site at 0 and 30 minutes and 2 and 5 hours after the initiation of infusion. To ensure the infused site had not shifted during the infusion patent blue was infused 30 minutes before the 2 and 5 hour sampling times.

After infusion was complete, the wound was closed with sutures and the animal dosed with antibiotics. Animals were recovered under supervision.

7.2.3 Preparation of plasmid DNA

The origin of plasmid DNA used for cDNA probes was as follows. pGLUT1 and pGLUT4 were obtained from the American Type Culture Collection (ATCC, 12301 Parklawn Drive, Rockville, Maryland 20852, USA). Information from ATTC accompanying the plasmids commented that pGLUT1 was derived from a human hepatoma HepG2 cell line (Mueckler *et al.*, 1985, Sarkar *et al.*, 1988) and consisted of a 2.47 kb insert cloned into pGEM-3 while pGLUT4 was derived from human jejunum and consisted of a 2 kb insert cloned into

pGEM-4Z (Fukumoto *et al.*, 1989). The pIR and pIGF-1 plasmids were obtained from Dr Jeff Johnson, of the Hormone Research Institute, University of California, San Francisco (UCSF), USA. They were both cloned from human placenta into pBluescript KS+. The insulin receptor cDNA is 4723 bp in length (Ebina *et al.*, 1985) while the IGF-1 receptor is 4975 kb long (Ullrich *et al., 1986*). The cDNA for rpS6 was a gift from Professor George Mackie of the Molecular Biology Laboratory of the University of Western Ontario. The 813 bp cDNA (Lott and Mackie, 1988) originated from human placenta and was cloned into pSP65. The B-actin plasmid was a gift from Dr Peter George of the Christchurch Medical School. It consisted of a 2 kb insert cloned into pGEM-3 (Gunning *et al.*, 1983).

Plasmid DNA was received either as a bacterial culture (GLUT1, GLUT4, insulin and IGF-1 receptors) or as plasmid DNA (rpS6, and B-actin). When not received as a culture, plasmids were first transformed into DH5α cells (appendix XII). Plasmid DNA was recovered according to appendix XIII or XIV (depending on quantity required).

Restriction maps corresponding to these plasmids were derived from the literature (appendix X). These restriction maps were tested once plasmid stocks had been prepared. Results are shown in appendix X.

7.2.4 Isolation of inserts from plasmid DNA

20-25 µg of plasmid DNA was digested with the appropriate restriction enzyme(s) for 2-3 hours at 37 ^{0}C. Insert and vector were separated on a 1% agarose gel prepared with wide tooth comb. The desired band was cut from the gel and the DNA recovered using a Bio-Rad Prep-A-Gene kit according to manufacturers recommendations. This kit initially solubilized the agarose gel slice releasing the DNA which is subsequently adsorbed onto a glass matrix. After six washes the DNA is eluted off the matrix with a low salt buffer.

7.2.5 DNA extraction

7.2.5.1 Human

Human DNA was extracted by a method adapted from Hickford (1989). Two 10 ml heparinized tubes of frozen blood were thawed at room temperature (freezing prior to extraction is essential). The contents were poured into a 50 ml Falcon tube and the

original tubes were washed with ice cold 75 mM KCl/50 mM EDTA (KCl) to remove leucocytes still adhering to the glass. Washings were subsequently also transferred to the Falcon tube. The volume was made up to 50 mls with KCl, and the tubes were centrifuged at 2500 rpm for 10 mins at 4 °C. The pellet was washed twice with KCl (with an intermittent centrifugation step at 2500 rpm for 10 mins at 4 °C) and twice in ice cold PBS/50 mM Na_2EDTA (with an intermittent centrifugation step of 2000 rpm for 10 mins at 4 °C). The pellet was resuspended in 1 ml of 0.9% NaCl, before the addition of 8 mls Adelaide B buffer and 1 ml 10 % SDS. Following thorough mixing, 300 µl of 10 mg/ml Proteinase K was added and the reaction incubated at 55 °C overnight to digest protein. After incubation, the tube was placed on ice and 2 mls of 5 M $NaClO_4$ was added. After mixing, the solution was extracted once with phenol, once with phenol: chloroform: isoamyl alcohol (25:24:1), and twice with chloroform. The aqueous phase was transferred to a new tube and sodium acetate was added to a final concentration of 300 mM prior to the addition of 2.5 volumes of absolute ethanol. Precipitated DNA was spooled out on a sealed glass capillary tube (fashioned into a hook) and washed overnight in 70% ethanol. DNA was air dried on a glass hook and resuspended in 1 x TE.

7.2.5.2 Rodent

A 3 mm^3 piece of liver tissue was digested in 0.7 ml of Pronase solution (1 X SET, 1 % SDS, 0.5 mg/ml Pronase) overnight at 37 °C. The digest was extracted once with 0.8 ml phenol:chloroform:isoamyl alcohol (25:24:1), and the aqueous layer transferred to a fresh tube. The DNA was precipitated with a 2.5x volume of absolute ethanol and washed in 70% ethanol before resuspension in 0.2 mls 1 x TE. RNA was removed by RNAse digestion at 37 °C for 30 minutes. The DNA was reprecipitated by adding 1/10th of volume of 3 M sodium acetate (pH 5.2) and 2.5x volumes of absolute ethanol. The DNA was pelleted, washed in 70% ethanol and resuspended in 1 x TE.

7.2.5.3 Sheep

Sheep DNA was extracted by a method adapted from Montgomery and Sise (1990). 15 mls of blood (collected into a heparinized tube) was made up to 50 mls using ice-cold RBC lysing solution in a Falcon tube. Tubes were mixed and left on ice for 20 minutes while lysis took place, prior to centrifugation at 3500 rpm for 10 minutes at 4 °C. The lysed material was decanted off leaving a pink pellet of white cells. The pellet was washed twice with ice cold TBS and centrifuged at 1000 rpm for 10 minutes at 4 °C. After

decanting off the remaining TBS, white cells were resuspended in 9 mls of ice cold 1 x TE. After the addition of 500 µl 0.5M Na_2EDTA, 75 µl 10 mg/ml proteinase K and 500 µl 10% SDS, tubes were incubated at 50 °C for 1.5 hours. This was followed by the addition of 4.3 mls saturated (5M) NaCl (after which tubes were shaken vigorously for 60 seconds) and centrifugation at 2500 rpm, 10 mins 4 °C. The supernatant was decanted off to a new tube. DNA was precipitated by the addition of 2 volumes of ice cold ethanol, after which it was spooled out on a sealed glass capillary tube (melted into a hook) and washed overnight in 70 % ethanol. DNA was air dried on the hook and resuspended in water.

7.2.6 RNA extraction

Follicles were scraped from the Dentimex strip immersed in liquid nitrogen straight into solution D using a sterile scalpel blade. Wool follicles and all other tissues were extracted using the acid guanidium thiocyanate method (Chomczynski and Sacchi, 1987, appendix XI). Modifications consisted of a more lengthy (overnight) isopropanol precipitation at -20°C and the removal of adhering droplets of moisture from the wall of the tube with a sterile cotton bud before the final resuspension step rather than using a Speedyvac to evaporate off remaining ethanol. RNA sometime needed as much as 2 days on ice to resuspend.

7.2.7 Southern hybridization

7.2.7.1 Sample preparation

10 µg of genomic DNA was digested overnight at 37 °C in a total reaction volume of 40 µl. The reaction consisted of 40 U enzyme (except for BamH I digests where 80 U were used), 4 µl 10 X reaction buffer (as received with the enzyme), 0.05 M spermidine (2 µl of a 0.1 M stock) and sterile dH_2O up to volume. The prepared digests were mixed by flicking the tubes followed by a brief centrifugation step to move all reagents to the bottom of the tube before incubation. Once digested, samples were loaded for agarose gel electrophoresis after the addition of 1/10th volume of loading dye.

7.2.7.2 Preparation of positive controls

1x, 10x and 100 x copy controls were prepared from cDNAs for candidate genes. This was achieved by calculating the number of chromosomes present in 10 µg genomic DNA (assuming an average of 4.6 pg DNA/cell, with each chromosome weighing 2.3 pg). This figure allowed the amount of each cDNA required to give a 1x copy control to be calculated (cDNA length/Avagodro's number x average molecular weight of a base x no. of chromosomes in 10 µg genomic DNA). The amount of 1 copy 'equivalent' was multiplied by 10 to give the 10x copy control and by 100 to give the 100x copy control.

7.2.7.3 Agarose gel electrophoresis (non-denaturing) and blotting procedure

Samples were electrophoresed on a 0.8 % agarose gel for 16 hours at 25 V and 20 °C using 1 x TBE containing 0.05 µg/ml EtBr as the buffer. On completion of the run the gel was photographed and transferred onto Hybond N+ by an alkaline capillary blot according to the manufacturers recommendations.

7.2.7.4 Hybridization

Membranes were sealed in heavy plastic bags and pre-hybridized for 3 hours while submerged in a 60 ^{0}C waterbath. The pre-hybridization solution consisted of 5 x SSPE, 0.5 % SDS, 5 x Denhardt's and 10 µg/ml denatured sheared salmon sperm DNA, amounting to a total volume of 25 mls. Twenty-five ng of the cDNA probe was radioactively labelled to a specific activity of 5-10 x 10^8 cpm/ug using a Ready-to-Go (BioRad, BioRad Laboratories PTY LTD) labelling kit according to the manufacturer's protocol. Incorporation of label was assessed by spotting 1 µl of the reaction mixture on a TLC plate and running the samples up the plate for approximately 7 cm in a solvent consisting of 750 mM KH_2PO_4. The plate was air-dried and exposed to X-ray film for 10 minutes at room temperature and incorporation assessed visually by comparison to a lane of pure radiolabelled dCTP. Incorporation was generally around 70%. Unincorporated nucleotides were removed by spinning the reaction through a G50 Sephadex column. This column was prepared by plugging a 1 ml syringe with glass wool and filling it with a Sephadex slurry which consisted of G50 swollen in 1 x TE. The column was drained for 10 min before loading. Separation of the loaded sample was achieved by centrifugation at 1200 rpm for 5 minutes which retrieved the labelled cDNA while unincorporated nucleotides remained in the column.

Hybridization reactions were prepared by adding 15 ng of labelled cDNA (3-6 x 10^8 cpm) to each membrane and mixing the solutions quickly and thoroughly by rolling the mixture around the bag with a glass pipette. The bags were incubated for 16 hours while submerged in a 60 ^{0}C water bath. Following hybridization, the membranes were washed twice in 2 x SSC, 0.1 % SDS for 15 minutes at room temperature followed by two 20 minute washes at 60 ^{O}C, also in 2 x SSC and 0.1 % SDS. The membranes were blotted dry and sealed in thin-walled plastic bags for autoradiography.

7.2.8 Northern Hybridization

7.2.8.1 Sample preparations

Samples were prepared by resuspending 10 µg of total RNA in 7 µl of ddH_2O. This dilution was added to 23 µl of denaturing buffer (500 µl formamide 100 µl MOPS, 160 µl formaldehyde, 40 µl EtBr (1 mg/ml) and 120 µl loading dye). Samples were denatured at 65 ^{0}C for 10 minutes and cooled on ice for 5 minutes before loading.

7.2.8.2 Preparation of positive controls

10x, 100x, 1000x and 10 000x copy control were prepared using the cDNAs for the candidate genes. It was assumed that each cell contained 13 pg of total RNA (an estimate for Hela cells). Given that a base has an average Mr of 274.25 g, it was possible to subsequently calculate the number of bases of total RNA per cell using the Avagadro's number (2.54 x 19E7 kb).

To work out how much cDNA was required for each lane, the length of each cDNA was divided by the number of bases of total RNA/cell and multiplied by the amount of sample loaded in each lane (10 µg). Finally this figure was halfed because cDNA gives 2 strands able to hybridize to the probe whereas RNA is single-stranded.

7.2.8.3 Agarose gel electrophoresis (denaturing) and blotting procedure

A 1.2 % denaturing gel was prepared by dissolving 1.8 g agarose into 110 mls of DEPC

treated ddH_20 while heated by microwave oven. Once the gel had cooled to 50 °C, 15 mls of 10 X MOPS, and 27 mls of 40% w/v formaldehyde were added. After thorough mixing the gel was cast. After loading the gel was electrophoresed in 1 x MOPS (DEPC treated) overnight at a constant voltage of 25 V. The following morning the gel was photographed and transferred onto Hybond N+ overnight using a neutral capillary blot with 20 x SSC. After the transfer, the membrane was photographed under UV illumination using Polaroid 665 film to provide both a positive and a negative image. The negative was used for normalisation of the lanes by densitometry in addition to B-actin hybridization if required. RNA was fixed onto the membrane by baking at 80 °C for 2 hours.

7.2.8.4 Hybridization

Membranes were sealed in heavy plastic and pre-hybridized for 3 hours submerged in a 50 °C waterbath. The pre-hybridization solution consisted of 5 x SSPE, 0.5 % SDS, 5 x Denhardt's, 10% dextran sulphate and 10 µg/ml denatured salmon sperm DNA, amounting to a total volume of 25 mls. Radiolabelled cDNA's were prepared as described in section 7.2.4.4. Six nanograms of radiolabelled cDNA (2.5×10^8 cpm) was added to each membrane and the solutions mixed by rolling the mixture around the bag with a glass pipette. The bags were incubated for 16 hours while submerged in water in a 50°C water bath. Following hybridization, the membranes were washed twice in 2 x SSC, 0.1 % SDS for 15 minutes at room temperature followed by two 20 minute washes at 50 °C, also in 2 x SSC and 0.1 % SDS. The membranes were blotted dry and sealed in thin-walled plastic bags for autoradiography.

7.2.9 Autoradiography and stripping of membranes

Autoradiographs were developed in cassettes with intensifying screens for 2 days to 5 weeks. After autoradiography the membranes were stripped by lying them flat in a plastic container and immersing them in a boiling solution of 1% SDS. Membranes were left to cool to room temperature, drip-dried, sealed in plastic and stored at -20 °C until further use.

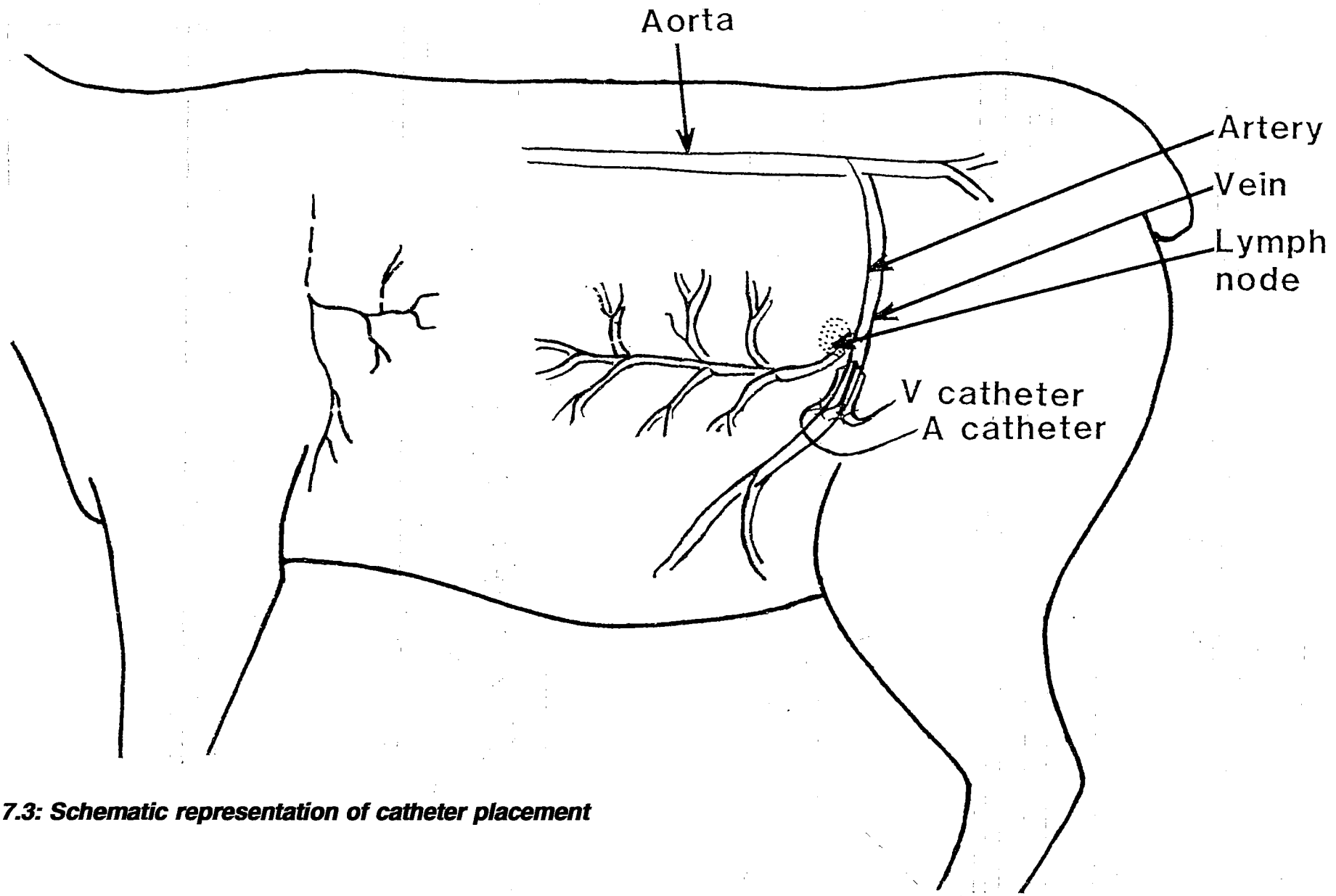


Figure 7.3: Schematic representation of catheter placement

7.3 Results

7.3.1 Insulin infusion

Chronic (5 hour) infusion of insulin into an isolated skin patch on the flank allowed wool follicle gene expression to be compared between the patch and a control area on the shoulder. Three high and three low wool strength animals were studied. The wool follicle RNA was pooled for the 3 animals in each group at each sampling time for control and infused wool follicle samples in order to reduce between animal variation and to ensure enough RNA was available for analysis.

Plasma insulin was measured both systemically (via the jugular vein) and locally at the site of infusion (from venous outflow from the patch). Those insulin values obtained from blood collected from the jugular were used for reference insulin (base level) values. Base level values showed a surprising amount of variation during the infusion but averaged around 13.6 uU/ml (n = 24, Tables 7.2-7.8). Animals in the low wool strength group showed patch insulin values of 44-50 μU above base levels. The high wool strength animals had insulin levels of 36-60 μU above base levels. Such increases are above those observed in glucose tolerance tests of the subset rams on 3 separate occasions. In the latter experiment an average rise of 35 μU above base levels was generally observed 10-20 minutes after administration of an intravenous dose of glucose, (data not shown). This suggests that the infused patch was exposed to insulin levels which equated to high physiological levels for sheep.

It was intended to raise insulin levels approximately 100 uU/ml above base levels. Consequently 1200 μU of insulin was supplied to the patch per minute on the assumption that blood flow through the patch was approximately 12 mls/minute (Harris *et al.*, 1988, 1989). Unfortunately outflow from the patch showed insulin levels much lower than this. It can therefore be concluded that blood flow was greater than 12 mls/min, or alternatively, significant amounts of insulin were bound by tissues within the 'patch', since insulin levels within the infusate were indeed found to be around 1200 μU/ml.

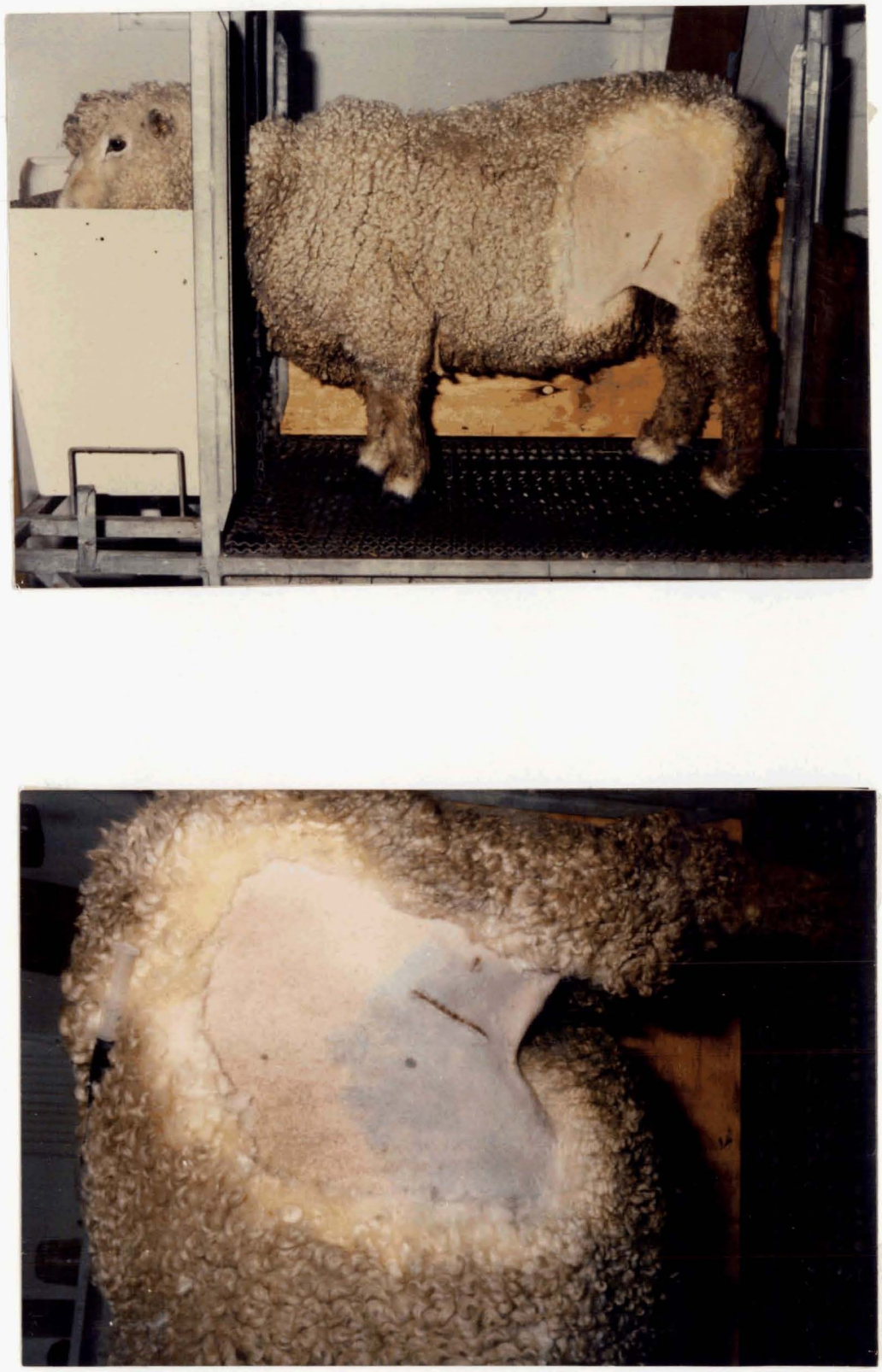


Figure 7.4: Verification of catheter patency of the isolated skin patch. The patch is shown before (top) and after (below) infusion of the radio-opaque dye Patent Blue.

Tag 352	t=0	t=30 m	t=2 h	t=5 h	Average
Patch	4.11	52.33	68.44	43.78	54.85
Systemic	3.16	4.07	11.27	7.40	6.48
Difference	0.95	48.26	57.17	36.38	48.37

Tag 439	t=0	t=30 m	t=2 h	t=5 h	**Average**
Patch	9.82	78.13	41.08	59.51	59.57
Systemic	10.85	11.65	12.22	26.71	15.36
Difference	-1.03	66.48	28.86	32.80	44.2

Tag 440	t=0	t=30 m	t=2 h	t=5 h	**Average**
Patch	8.72	69.81	60.72	99.39	76.64
Systemic	10.59	10.59	66.21	16.66	26.02
Difference	-1.87	59.22	-5.49	82.73	50.62

Tables 7.2 - 7.4: Isolated skin patch and systemic plasma insulin levels during the course of insulin infusion for the low wool strength animals.

Whole body and patch plasma insulin levels (μU/ml) are shown at times 0, 30 minutes, 2 hours and 5 hours. The row entitled 'difference' represents the amount that patch plasma insulin levels were raised over the control (systemic) values. Patch and whole body average plasma insulin levels were calculated over t=30 min to t = 5 hours and t = 0 to t = 5 hours respectively.

Tag 415	t=0	t=30 m	t=2 h	t=5 h	**Average**
Patch	6.60	58.46	78.51	---	68.49
Systemic	10.93	12.74	7.56	---	10.41
Difference	-4.33	45.72	70.95	---	58.08

Tag 589	t=0	t=30 m	t=2 h	t=5 h	**Average**
Patch	3.47	47.22	38.90	44.64	43.59
Systemic	3.64	5.89	8.33	11.00	7.22
Difference	-0.17	41.33	30.57	33.64	36.37

Tag 641	t=0	t=30 m	t=2 h	t=5 h	**Average**
Patch	4.14	80.50	64.29	85.36	76.72
Systemic	5.28	6.92	8.67	44.42	16.32
Difference	-1.14	73.58	55.62	40.94	60.4

Tables 7.5 - 7.7: Isolated skin patch and systemic plasma insulin levels during the course of insulin infusion for the high wool strength animals.

Whole body and patch plasma insulin levels (μU/ml) are shown at times 0, 30 minutes, 2 hours and 5 hours. The row entitled 'difference' represents the amount that patch plasma insulin levels were raised over the control (systemic) values. Patch and whole body average plasma insulin levels were calculated over t=30 min to t = 5 hours and t = 0 to t = 5 hours respectively.

7.3.2 Gene expression studies

For each candidate gene the species specificity of its cDNA was determined by hybridizing the human cDNA to restriction digested human, rodent (rat or mouse) and ovine DNA. This allowed the authenticity of the probe to be assessed (by comparing the patterns to previously published results), as well as confirming the cDNA's ability to hybridize to the DNA from other species. To further check the authenticity of the probes they were checked against the plasmid maps in appendix X by restriction mapping (also appendix X).

The expression of each candidate gene was investigated in a variety of ovine tissues equivalent to those in which expression of the candidate genes has previously been reported in rat and human studies. Finally expression of each gene was assessed in the presence of high physiological doses of insulin.

7.3.2.1 Glucose transporter 1 gene

It was not possible to match the mouse and human Southern hybridization patterns with those of others. Southern blotting of BamH I digested human DNA with a GLUT1 cDNA revealed a pattern which did not match the results of Shows *et al.* (1987) who reported fragments of 15.3, 12.1, and 7.8 kb with weak hybridization to a fourth fragment of 9.4 kb. Much smaller fragments of 1.8 - 3.5 kb were observed in this work (Figure 7.5) even after a repeat experiment carried out by another worker in the laboratory. Restriction mapping of pGLUT1 ascertained that it matched the restriction data issued by ATTC (appendix X). It is therefore not known what is responsible for the inconsistency between results. Some considerations are that the human, rat and sheep DNA used in this experiment was also used for the blots prepared for IR, IGF-IR, rpS6 and B-actin hybridization. Given that these hybridization produced hybridization patterns comparable to those published by others discounts DNA degradation as responsible for producing smaller fragments for the GLUT1 Southern hybridization. The most feasible explanation is that restriction digestion or hybridization conditions in this work were different to those used by Shows *et al.*, (1987) thereby either causing the restriction enzyme to cut at different sites or the cDNA probe to hybridize to different fragments.

Poor hybridization prevented the mouse BamH I digest being compared with the results of Shows *et al.* (1987) who reported bands of 10.7, 3.5 and 3.1 kb for this digest. The lack of hybridization can in part be accounted for by loading differences but it may have been

compounded by a lack of homology. A good signal was achieved with hybridization to sheep DNA. A BamH I digest produced fragments of 10.5 and 5 kb while the Bgl II digest produced fragments of 7.4, 4.5 and 2.2 kb.

Northern blotting of skin, wool follicles, fat, muscle, brain and liver showed that the GLUT1 gene was expressed at low levels in skin and brain tissues (Figure 7.11). Copy number controls indicated that the transcript was expressed at 10-100 copies per cell. The size of the transcript, approximately 2.8 kb, is equivalent to that found in bovine (2.9 kb, Boado and Pardridge, 1990), human (Thorens *et al.*, 1990) and rat tissues (Sivitz *et al.*, 1989).

The effect of insulin infusion on GLUT1 gene expression was not ascertained as expression was not detected in this experiment despite the 10 copy control producing a strong signal (Figure 7.18). Although there are two sets of bands discernable, they are of a size corresponding to 18S and 28S ribosomal RNA and are therefore likely to represent non-specific background hybridization.

7.3.2.2 Glucose transporter 4 gene

Bell *et al.* (1989), reported that Southern blots of BamH I digested human and mouse genomic DNA produced a 9 and 11 kb band respectively. This work produced bands of 11.2 and 7.9 kb for BamH I digested human DNA and 11.2, 7.9 and 4.5 kb bands for BamH I mouse DNA (Figure 7.6). Restriction mapping of the GLUT4 fragment after excision from the vector was carried out to ensure the cDNA had the correct sequence as determined by the presence of previously published restriction sites (appendix X). The GLUT4 cDNA hybridized strongly to sheep DNA producing 11.2, 4.2 and 2.3 kb bands with a BamH I digested DNA.

Northern hybridization with a GLUT4 cDNA of ovine tissues produced a 4.4-4.6 kb band (Figure 7.12). A 3.5-3.6 kb (Thorens *et al.*, 1990) and 2.8 kb (Tordjman *et al.*, 1989) GLUT4 mRNA has been reported for human tissues and a 2.8 kb GLUT4 mRNA for rat tissue (Sivitz *et al.*, 1989, Thorens *et al.*, 1990). In our laboratory, Craigen (1994, personal communication) has shown the presence of a 2.2 and 5.4 kb band in ovine intestine tissues using the same GLUT4 cDNA as used in this work (Figure 7.13). It therefore appears that the 4.4-4.6 kb band observed in Figure 7.12 corresponds to non-specific hybridization to 28S rRNA and that no hybridization of GLUT4 cDNA occurred in any of the tissues studied.

Insulin infusion did not induce the expression of GLUT4 mRNA (Figure 7.19).

7.3.2.3 Insulin receptor gene

Southern hybridization of EcoR I digested human DNA produced bands of 13, 11, 5.8, 5.5, and 3.8 kb as described by other workers (Taylor *et al.*, 1990, Figure 7.7). The corresponding sheep EcoR I digest produced bands of approximately 13.3, 6.3, 4.2, 3.3 and 1.4 kb.

Northern hybridization did not detect the expression of the insulin receptor gene in any tissues examined, despite good signal being obtained with the 10 copy control (Figure 7.14). Although bands were observed at approximately 4.4-4.6 kb, human studies showing insulin receptor mRNA's of 5.2-10.5 kb (Ullrich *et al.*, 1986, Taylor *et al.*, 1992), would suggest that these smaller mRNAs correspond to 28S rRNA.

Although some differences in band intensity were observed between lanes, it is not thought that insulin infusion induced the expression of an insulin receptor mRNA. This is because the band is not of the expected size and the loading of the lanes (Figure 7.21) appears to explain differences in intensity.

7.3.2.4 Insulin like growth factor I receptor gene

Southern blots of EcoR I digested human DNA using an IGF-1 receptor cDNA gave bands of approximately 13, 6.5, 3.8 and 3.4 kb (Figure 7.8) which corresponds well to the results of Ullrich *et al.* (1986). An equivalent digest using sheep DNA produced bands of approximately 12 and 4.5 as well as many smaller bands on longer exposures.

Human studies have shown IGF-1 receptor mRNAs of 11 and 7 kb in length. Rat tissues yielded a single 11 kb IGF-1 receptor mRNA (Ullrich *et al.*, 1986). This work however only achieved a signal in the 4.4-4.6 kb region (Figure 7.15) which corresponds to the size of 28S rRNA. It therefore appears that the expression of IGF-1 receptor mRNA cannot be detected in the tissues examined given that a good signal was achieved with the 10 copy control.

Insulin infusion did not induce the expression of IGF-1 receptor mRNA (Figure 7.22).

7.3.2.5 Ribosomal protein S6 gene

Southern blotting of EcoRI and Hind III digested human DNA (Figure 7.9) produced banding patterns which were very similar to those described by Lott and Mackie (1988). The corresponding patterns for sheep DNA also involved numerous bands which are likely to represent the presence of multiple genes of pseudogenes (Lott and Mackie, 1988).

Northern blotting indicated that rpS6 mRNA is expressed in all the tissues examined (Figure 7.16), with the transcript size of 0.9-1 kb being of approximately the same size as rpsS6 mRNA found in human tissues (1.0 kb, Lott and Mackie, 1988). Copy number controls indicate that approximately 100 copies exist in each cell. Insulin infusion did not increase the level of rpS6 expression (Figure 7.24). Although some differences in intensity could be observed, these can be explained by relative loading in the lanes (Figure 7.23).

7.3.2.6 B-actin

Southern blotting of human DNA using the B-actin cDNA gave strong hybridization signals with human and sheep DNA (Figure 7.10).

Northern blotting using the B-actin cDNA was very successful with the autoradiography requiring a 2-3 day exposure time (Figure 7.17) compared to approximately 7 days exposure time for the rpS6 cDNA and 3-5 weeks exposure time for the GLUT1, GLUT4, IR and IGF-2IR cDNAs. Tissues specificity data showed that the cDNA had hybridized to a message slightly smaller in size in muscle tissue. It is likely that this is the result of cross-hybridization of the cDNA to α actin which is the dominant isoform found in muscle (Gunning *et al.*, 1983). Actin expression in response to an insulin infusion was not assessed as the lack of expression for the candidate genes as described meant that normalisation of the lanes was not required.

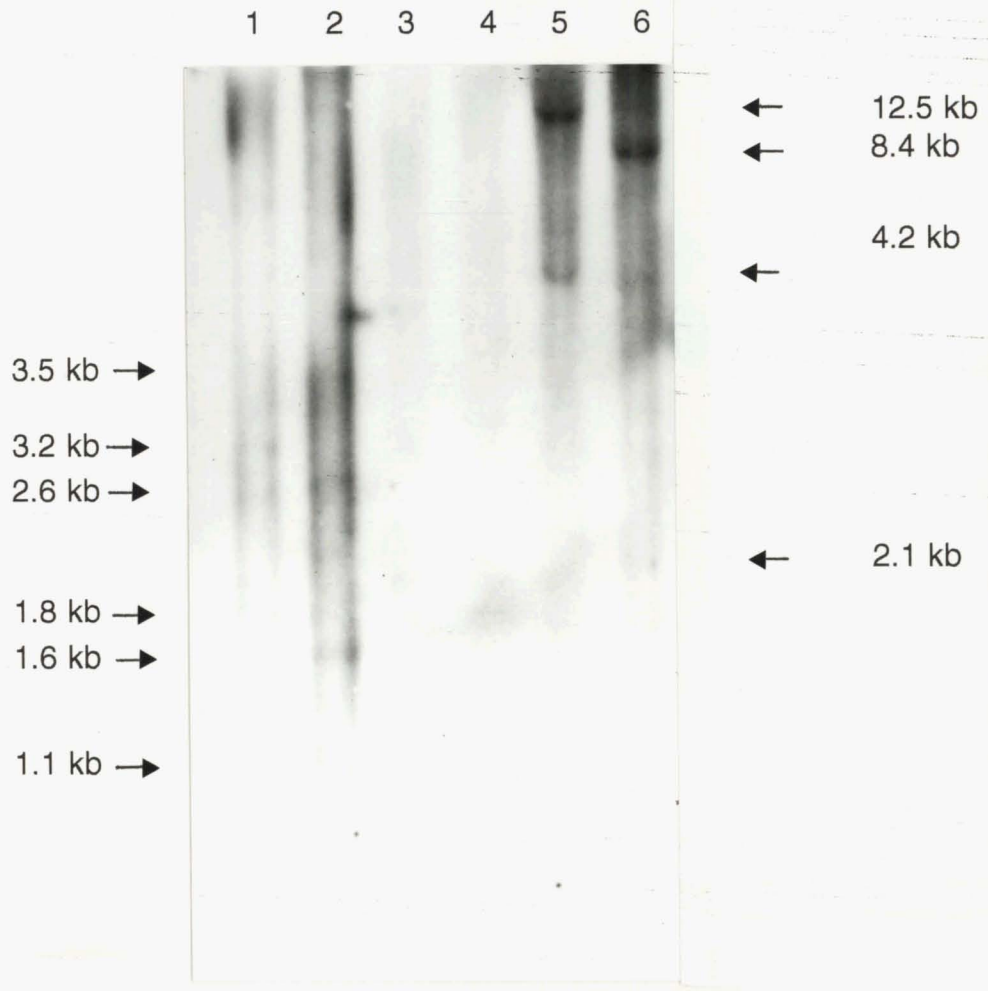


Figure 7.5: Southern blot using the human GLUT1 cDNA (Mueckler et al., 1985, Sarkar et al., 1988, Appendix X)

Lane 1) Human BamH I digest 2) Human Bgl II digest 3) Mouse BamH I digest 4) Mouse Bgl II digest 5) Sheep BamH I digest 6) Sheep Bgl II digest. 3 week exposure. Each lane represents 10µg of digested genomic DNA.

Figure 7.6: Southern blot using the human GLUT4 cDNA (Fukumoto et al., 1989, Appendix X).

Lane 1) Human BamH I digest 2) Human Hind III digest 3) Mouse BamH I digest 4) Mouse Hind III digest 5) Sheep BamH I digest 6) Sheep Hind III digest. 3 week exposure. Each lane represents 10µg of digested genomic DNA.

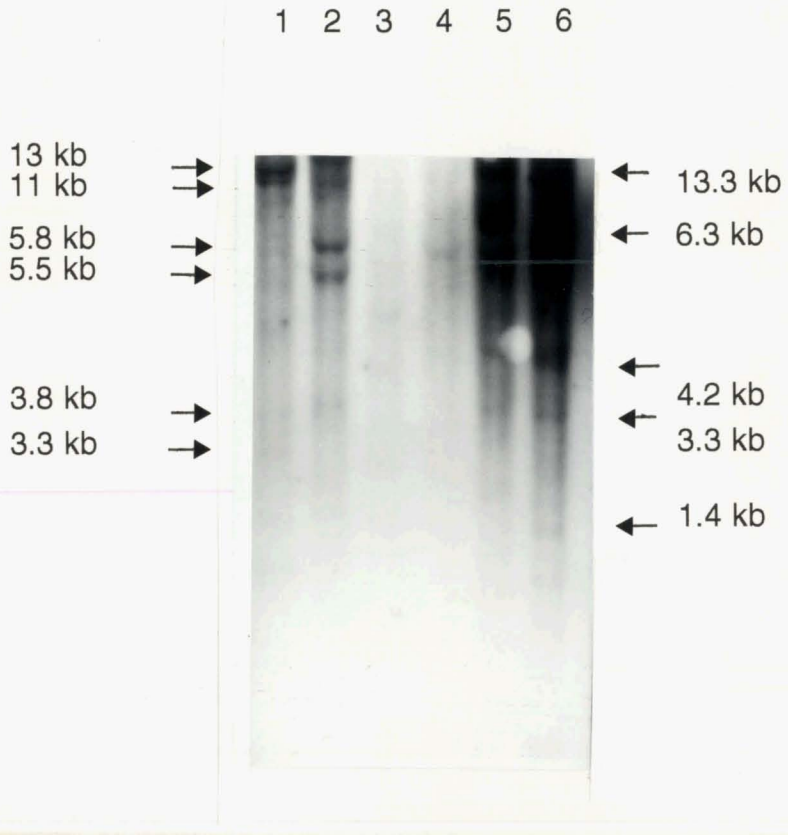


Figure 7.7: Southern blot using the human insulin receptor cDNA (Ebina et al., 1985, Appendix X)

Lane 1) Human Hind III digest 2) Human EcoR I digest 3) Mouse Hind III digest 4) Mouse EcoR I digest 5) Sheep Hind III digest 6) Sheep EcoR I digest. 2 week exposure. Each lane represents 10µg of digested genomic DNA.

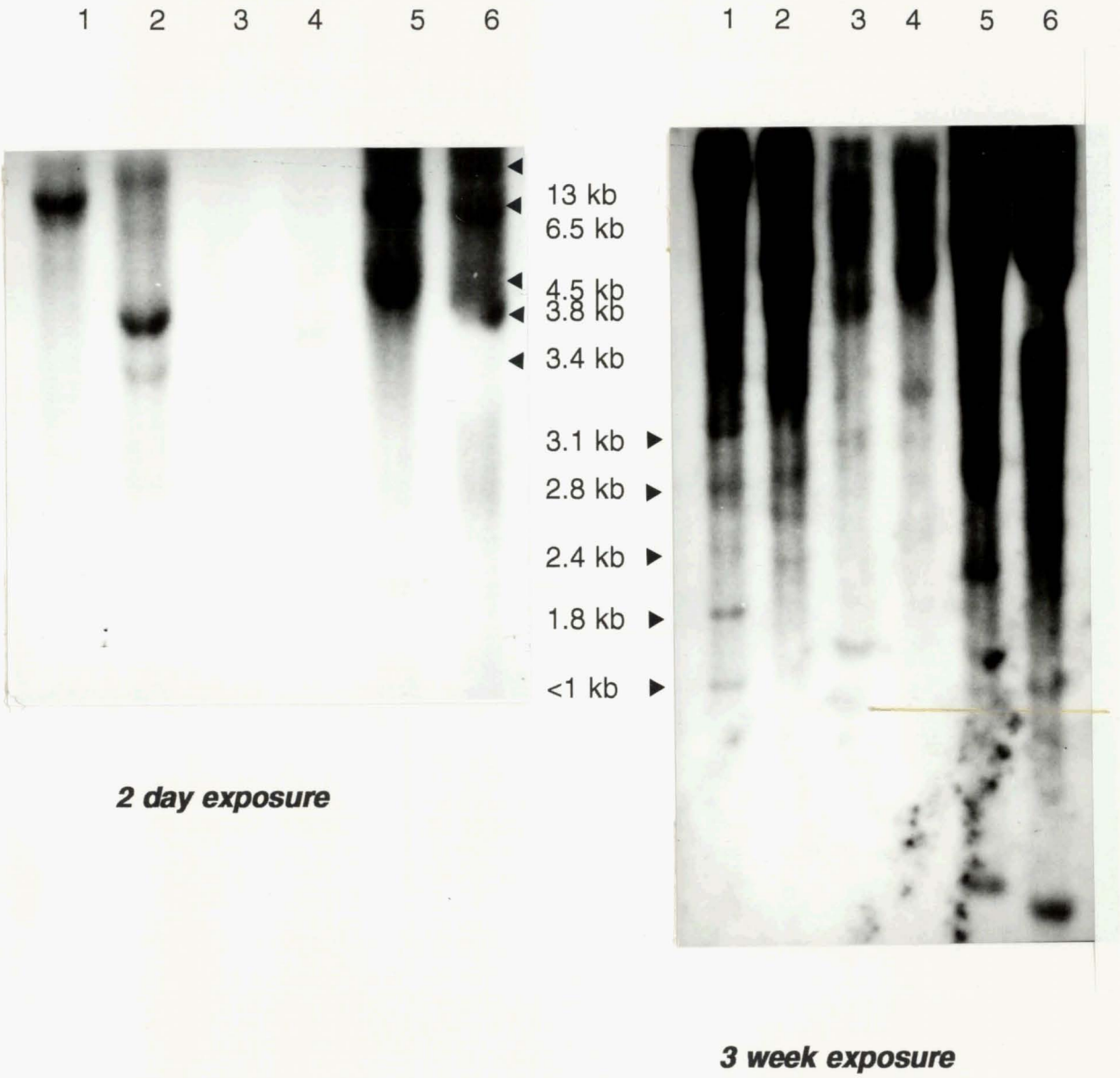


Figure 7.8: Southern blot using the human insulin-like growth factor I cDNA (Ullrich et al., 1986, Appendix X)

Lane 1) Human Hind III digest 2) Human EcoR I digest 3) Mouse Hind III digest 4) Mouse EcoR I digest 5) Sheep Hind III digest 6) Sheep EcoR I digest. 3 week exposure. Each lane represents 10 µg of digested genomic DNA.

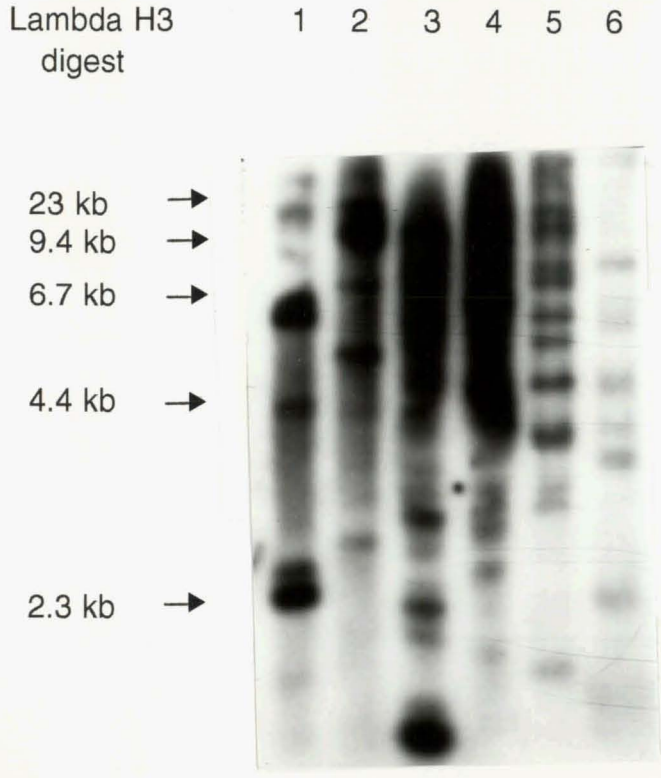


Figure 7.9: Southern blot using the human ribosomal protein S6 cDNA (Lott and Mackie, 1988, Appendix X).

Lane 1) Human Hind III digest 2) Human EcoR I digest 3) Rat Hind III digest 4) Rat EcoR I digest 5) Sheep Hind III digest 6) Sheep EcoR I digest. 6 day exposure. Each lane represents 10µg of genomic DNA.

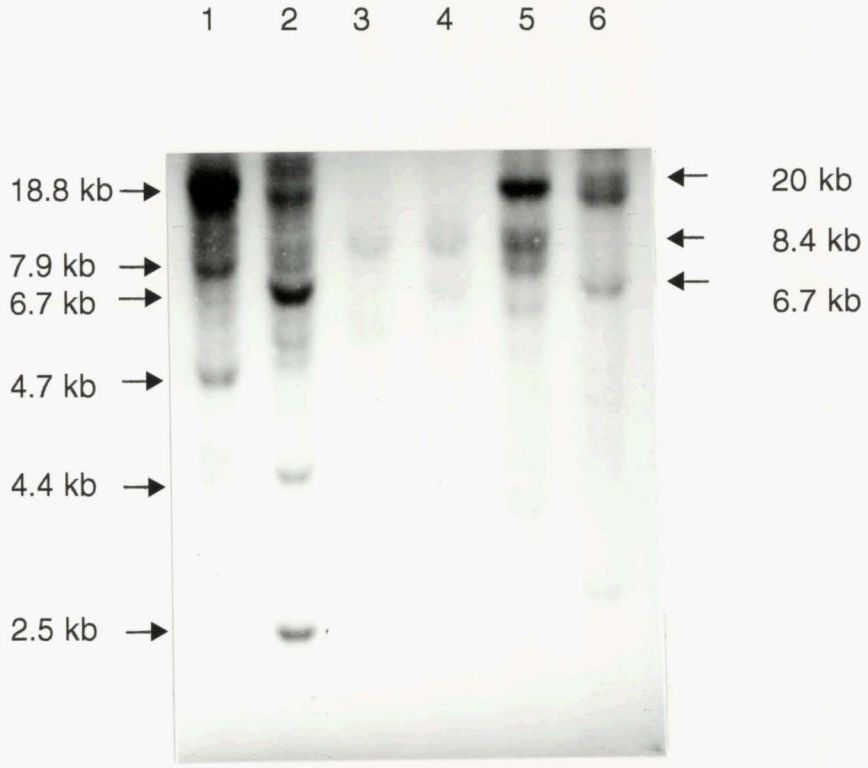


Figure 7.10: Southern blot using the human β-actin cDNA (Gunning et al., 1983)

Lane 1) Human Hind III digest 2) Human EcoR I digest 3) Mouse Hind III digest 4) Mouse EcoR I digest 5) Sheep Hind III digest 6) Sheep EcoR I digest. 2 week exposure. Each lane represents 10μg of genomic DNA.

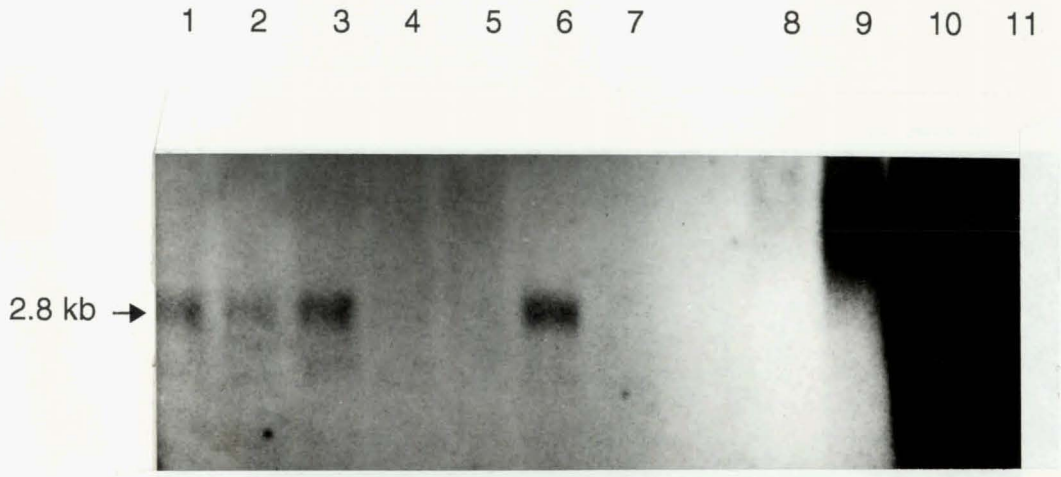


Figure 7.11: Tissue specificity of GLUT1 gene expression in ovine tissue (Hueckler et al., 1985, Sarkar et al., 1988, Appendix X).

Lane 1) skin 2) epilated skin 3) epilated wool follicles 4) sub-cutaneous fat 5) muscle 6) Brain 7) Liver 8) 10 copy control 9) 100 copy control 10) 1 000 copy control 11) 10 000 copy control. 3 week exposure. Lanes 1-7 contain 10µg of total RNA.

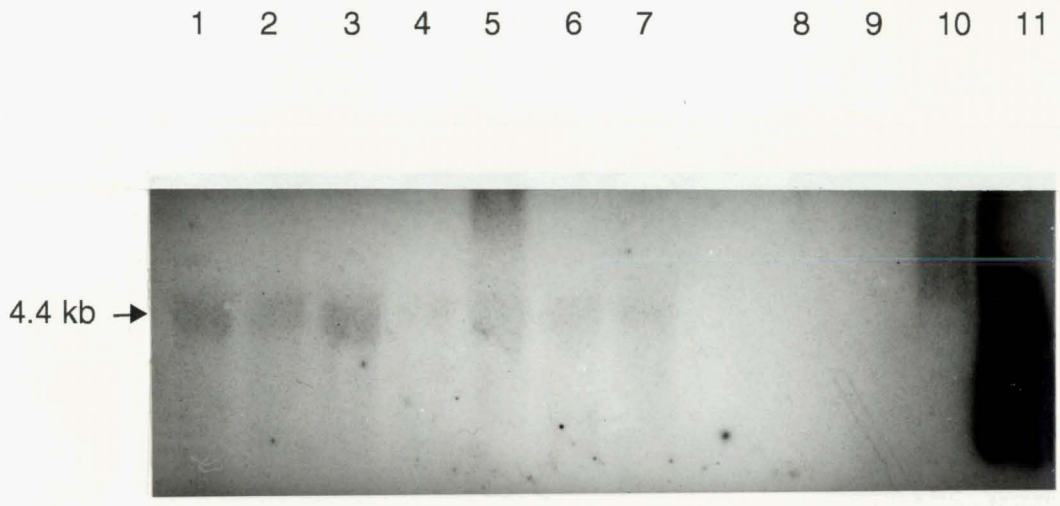


Figure 7.12: Tissue specificity of GLUT4 gene expression in ovine tissue (Fukumoto et al., 1989, Appendix X).

Lane 1) skin 2) epilated skin 3) epilated wool follicles 4) sub-cutaneous fat 5) muscle 6) Brain 7) Liver 8) 10 copy control 9) 100 copy control 10) 1 000 copy control 11) 10 000 copy control. 3 week exposure. Lanes 1-7 contain 10µg of total RNA.

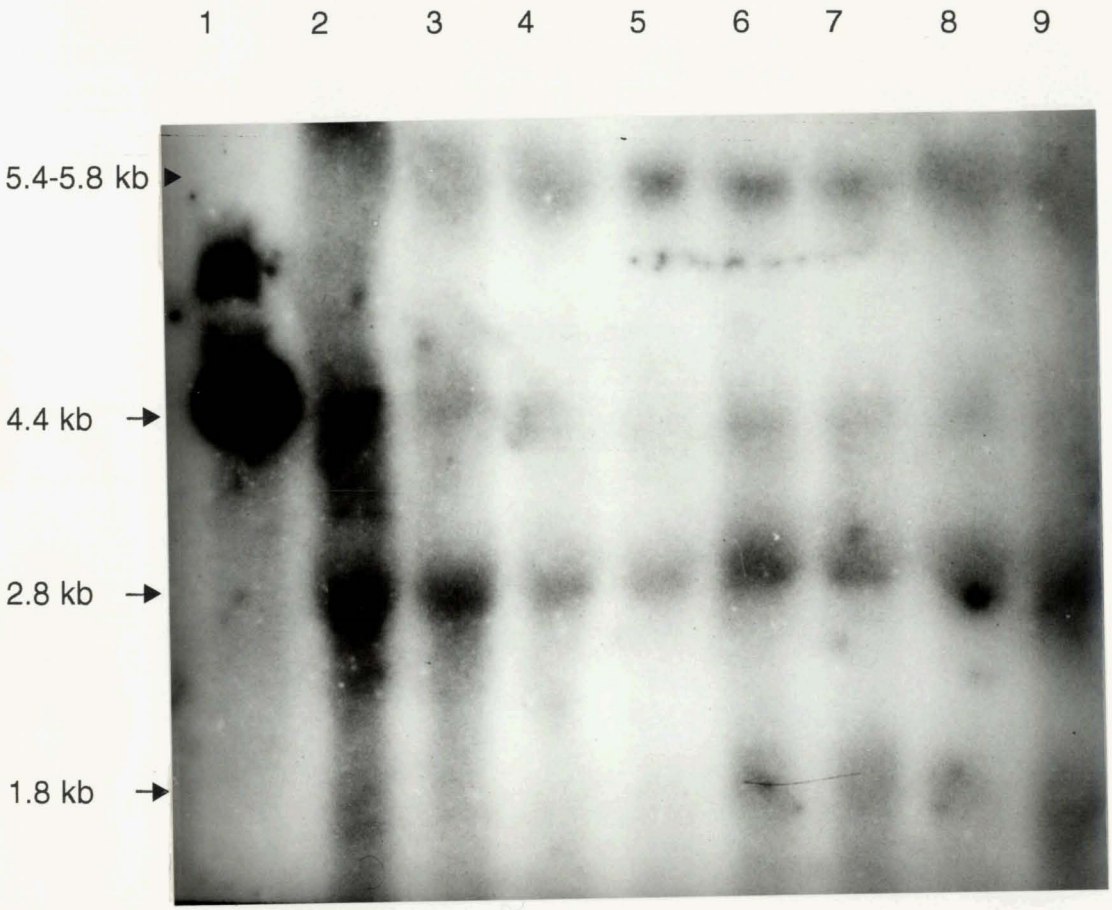


Figure 7.13: GLUT4 gene expression in ovine small intestine (with permission from M.C. Craigen, 1994, in preparation)

Lane 1) RNA Ladder (BRL) 2-9) animals 1-8. 6 week exposure. Lanes 2-9 contain 10µg of total RNA.

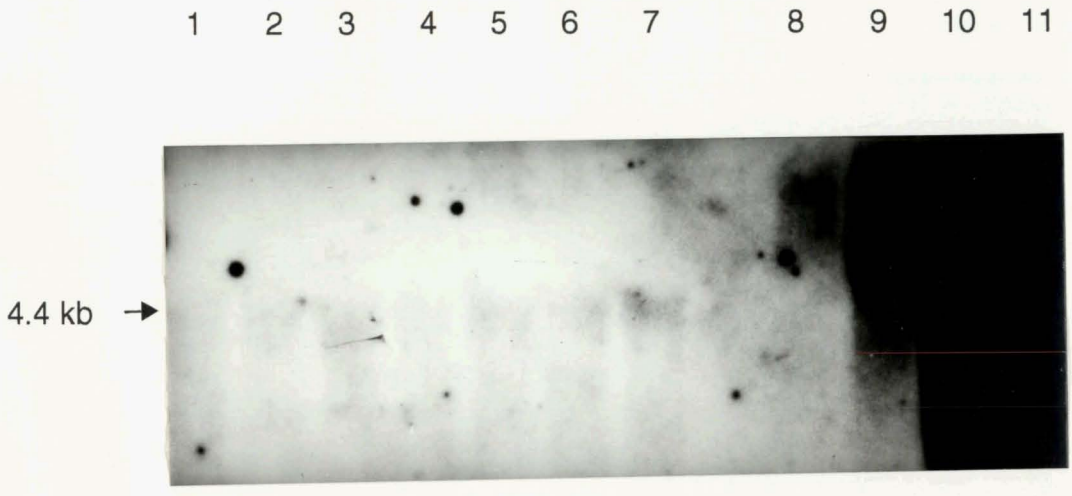


Figure 7.14: Tissue specificity of insulin receptor gene expression in ovine tissue (Ebina et al., 1985, Appendix X)

Lane 1) skin 2) epilated skin 3) epilated wool follicles 4) sub-cutaneous fat 5) muscle 6) Brain 7) Liver 8) 10 copy control 9) 100 copy control 10) 1 000 copy control 11) 10 000 copy control. 3 week exposure. Lanes 1-7 contain 10µg of total RNA.

Figure 7.15: Tissue specificity of insulin-like growth factor I expression in ovine tissue (Ullrich et al., 1986, Appendix X).

Lane 1) skin 2) epilated skin 3) epilated wool follicles 4) sub-cutaneous fat 5) muscle 6) Brain 7) Liver 8) 10 copy control 9) 100 copy control 10) 1 000 copy control 11) 10 000 copy control. 3 week exposure. Lanes 1-7 contain 10µg of total RNA.

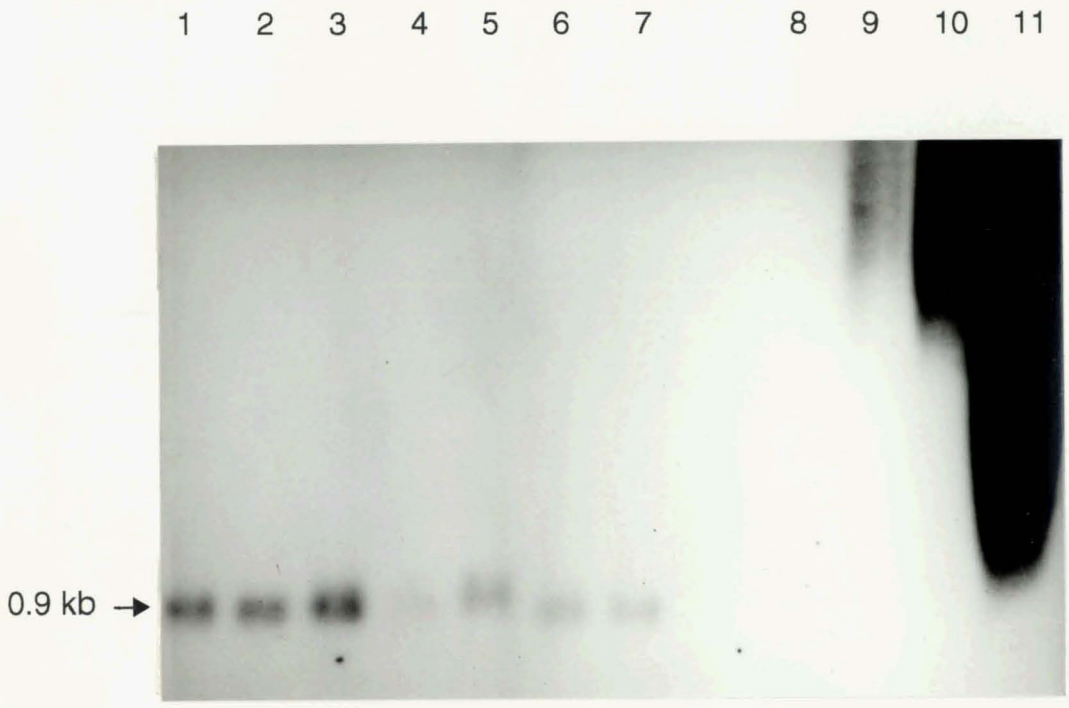


Figure 7.16: Tissue specificity of ribosomal protein S6 gene expression ovine tissue (Lott and Mackie, 1988, Appendix X).

Lane 1) skin 2) epilated skin 3) epilated wool follicles 4) sub-cutaneous fat 5) muscle 6) Brain 7) Liver 8) 10 copy control 9) 100 copy control 10) 1 000 copy control 11) 10 000 copy control. 3 week exposure. Lanes 1-7 contain 10µg of total RNA.

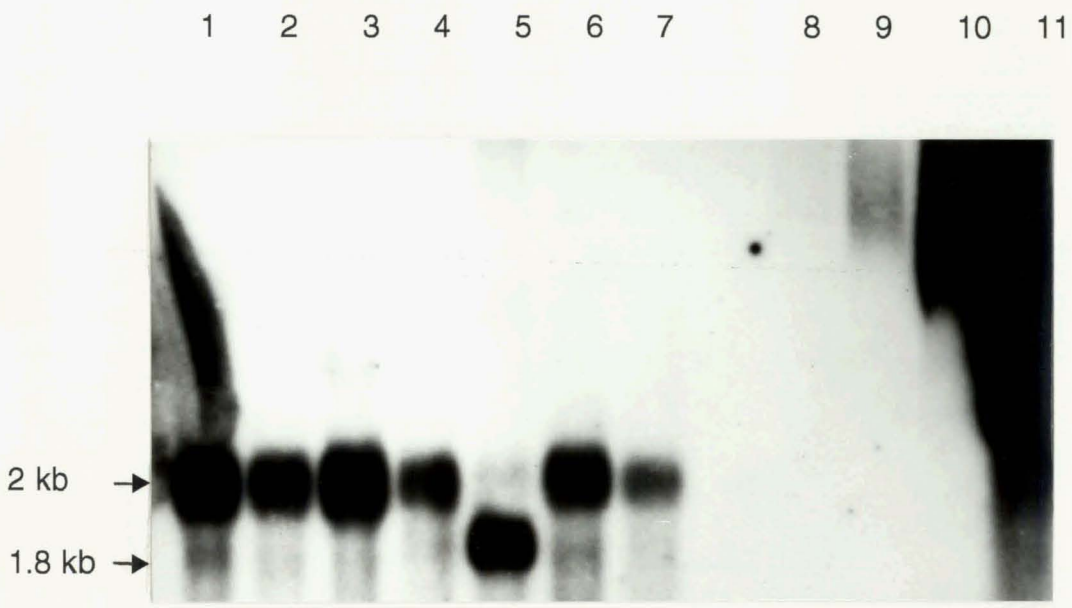
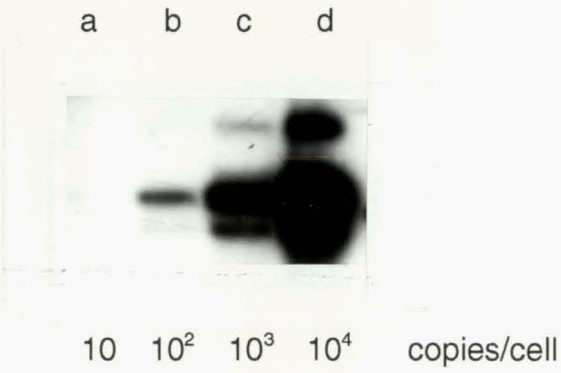


Figure 7.17: Tissue specificity of β-actin gene expression in ovine tissue (Gunning et al., 1983)

Lane 1) skin 2) epilated skin 3) epilated wool follicles 4) sub-cutaneous fat 5) muscle 6) Brain 7) Liver 8) 10 copy control 9) 100 copy control 10) 1 000 copy control 11) 10 000 copy control. 3 week exposure. Lanes 1-7 contain 10μg of total RNA.



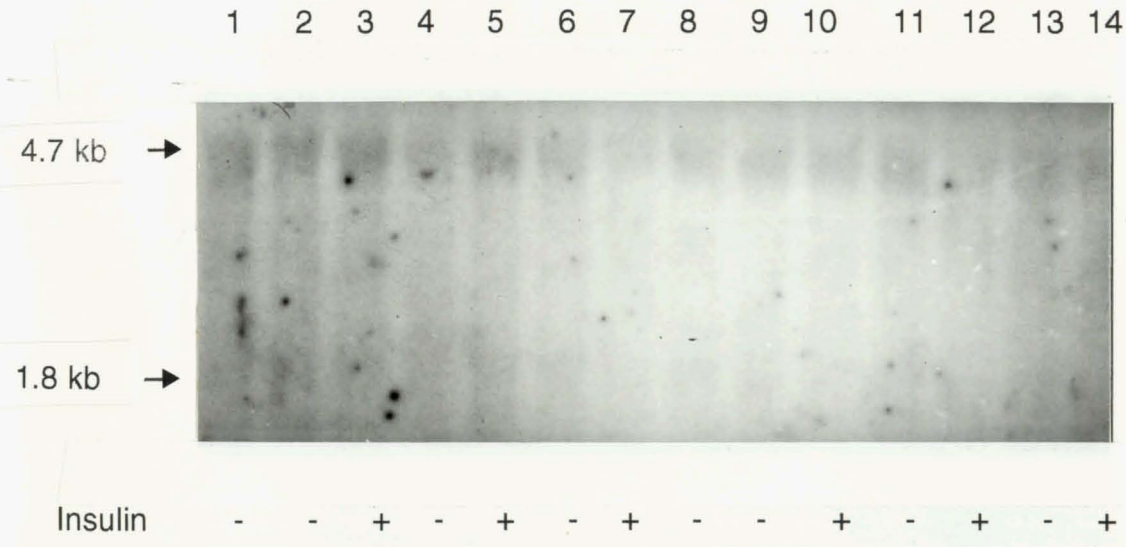
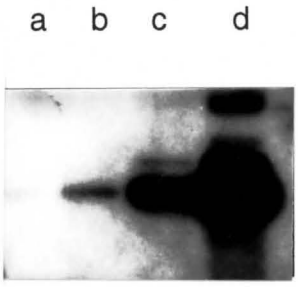


Figure 7.18: Effect of insulin infusion on GLUT1 gene expression in the wool follicle (Mueckler et al., 1985, Sarkar et al., 1988, Appendix X).

Lanes a-d) copy number controls. Lane **1)** LSS, t = 0, **2-3)** LSS, t = 30 minutes, **4-5)** LSS, t = 2 hours, **6-7)** LSS, t = 5 hours, **8)** HSS, t = 0, **9-10)** HSS, t = 30 minutes, **11-12)** HSS, t = 2 hours, **13-14)** HSS, t = 5 hours. **LSS** = low staple strength. **HSS** = high staple strength. 3 week exposure. Lanes 1-14 contain 10µg of total RNA.



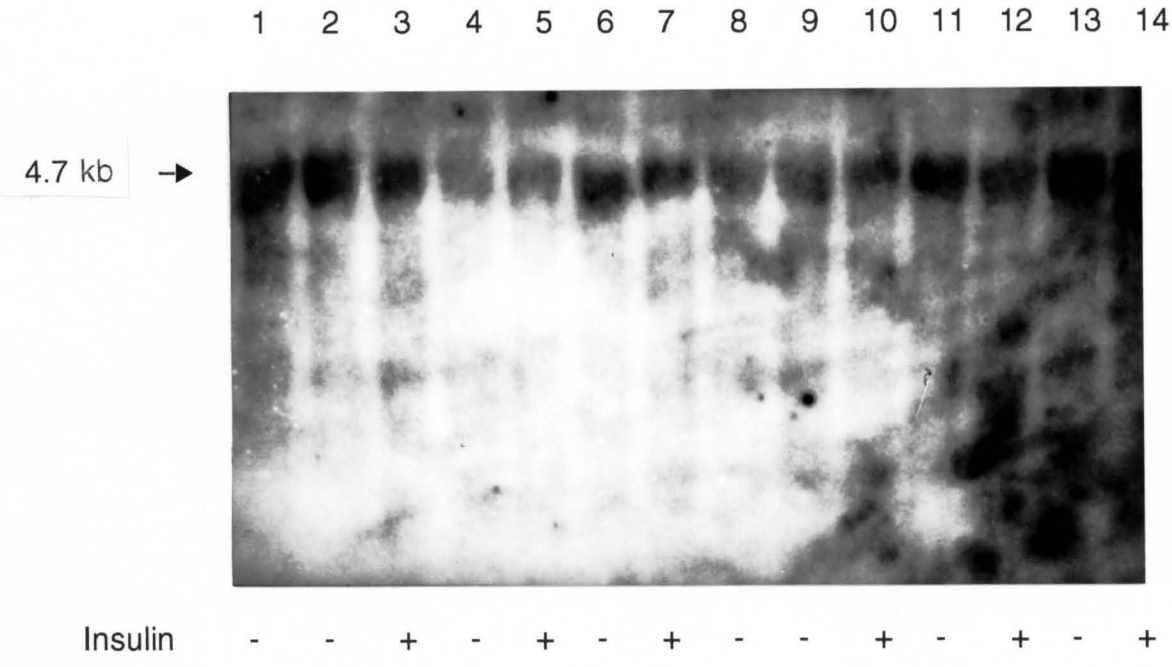


Figure 7.19: Effect of insulin infusion on GLUT4 gene expression in the wool follicle (Fukumoto et al., 1989, Appendix X).

Lanes a-d) copy number controls. Lane **1)** LSS, t = 0, **2-3)** LSS, t = 30 minutes, **4-5)** LSS, t = 2 hours, **6-7)** LSS, t = 5 hours, **8)** HSS, t = 0, **9-10)** HSS, t = 30 minutes, **11-12)** HSS, t = 2 hours, **13-14)** HSS, t = 5 hours. **LSS** = low staple strength. **HSS** = high staple strength. 3 week exposure. Lanes 1-14 contain 10µg of total RNA.

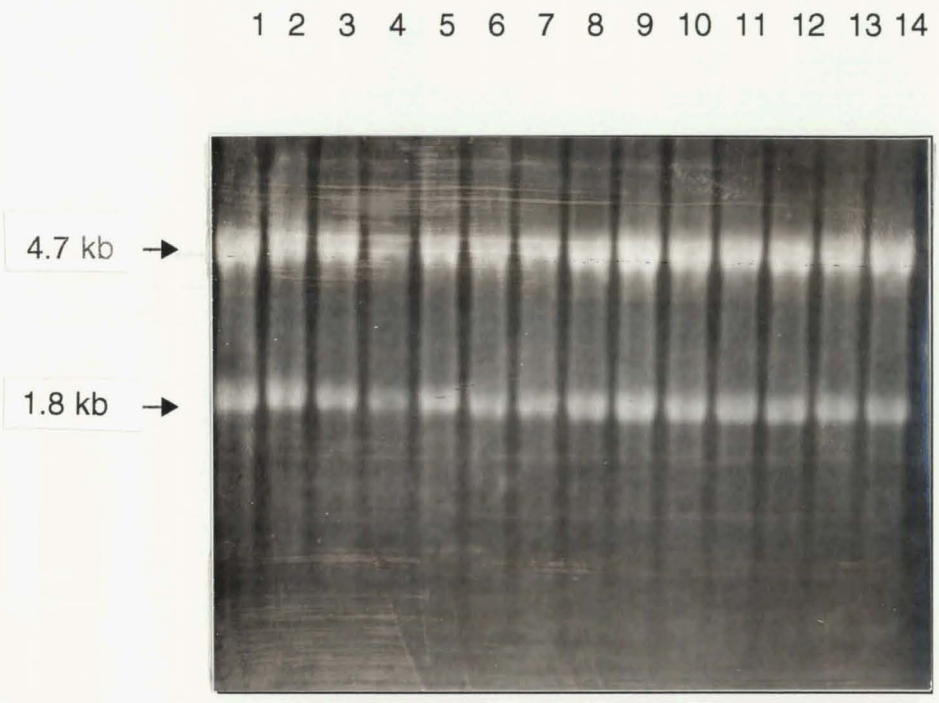


Figure 7.20: Relative transfer between samples of total RNA from the gel onto the membrane for the blot used for figure 7.21

Lanes 1-14 contain 10µg of total RNA.

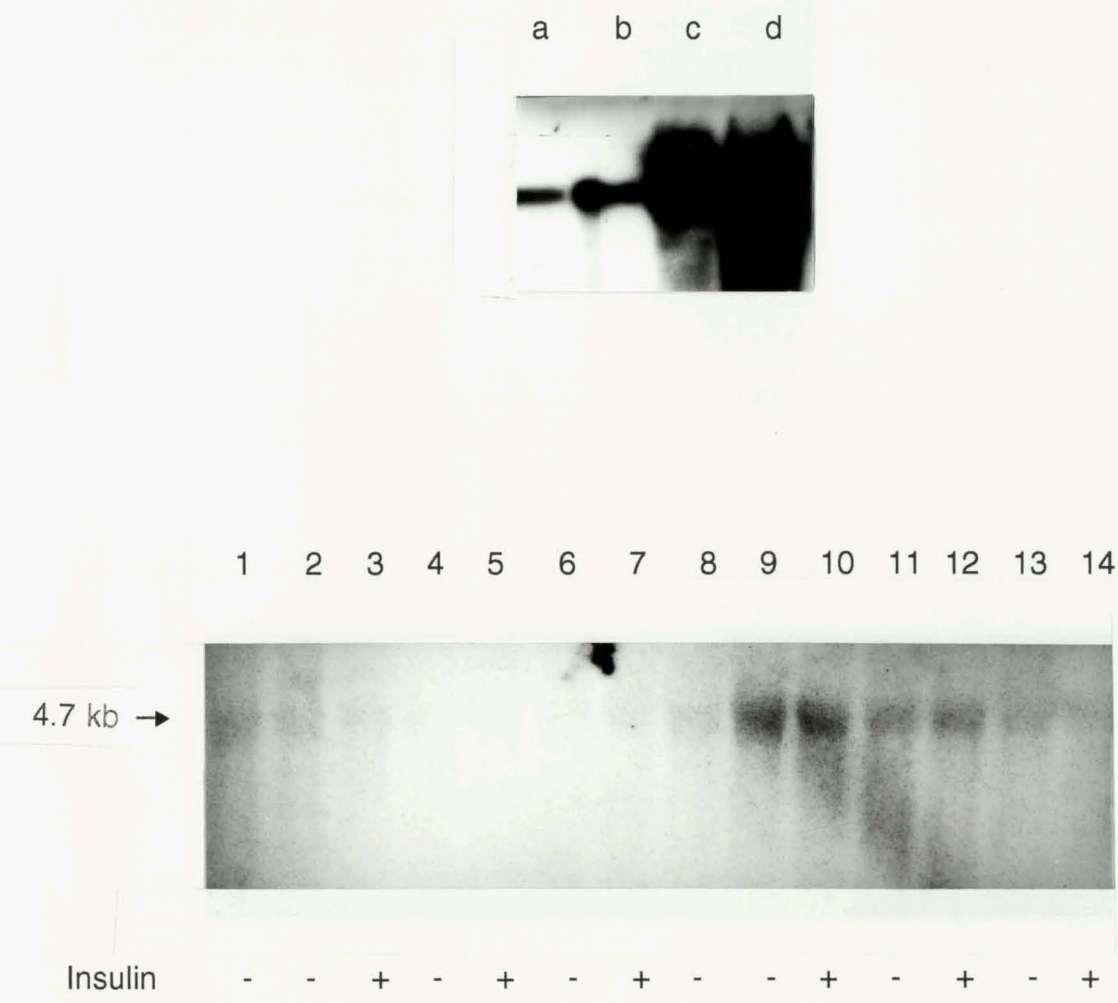
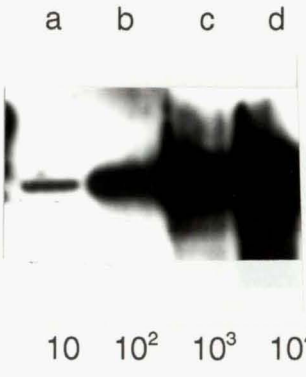


Figure 7.21: Effect of insulin infusion on insulin receptor gene expression in the wool follicle (Ebina et al., 1985, Appendix X).

Lanes a-d) copy number controls. Lane **1)** LSS, t = 0, **2-3)** LSS, t = 30 minutes, **4-5)** LSS, t = 2 hours, **6-7)** LSS, t = 5 hours, **8)** HSS, t = 0, **9-10)** HSS, t = 30 minutes, **11-12)** HSS, t = 2 hours, **13-14)** HSS, t = 5 hours. **LSS** = low staple strength. **HSS** = high staple strength. 3 week exposure. Lanes 1-14 contain 10µg of total RNA.



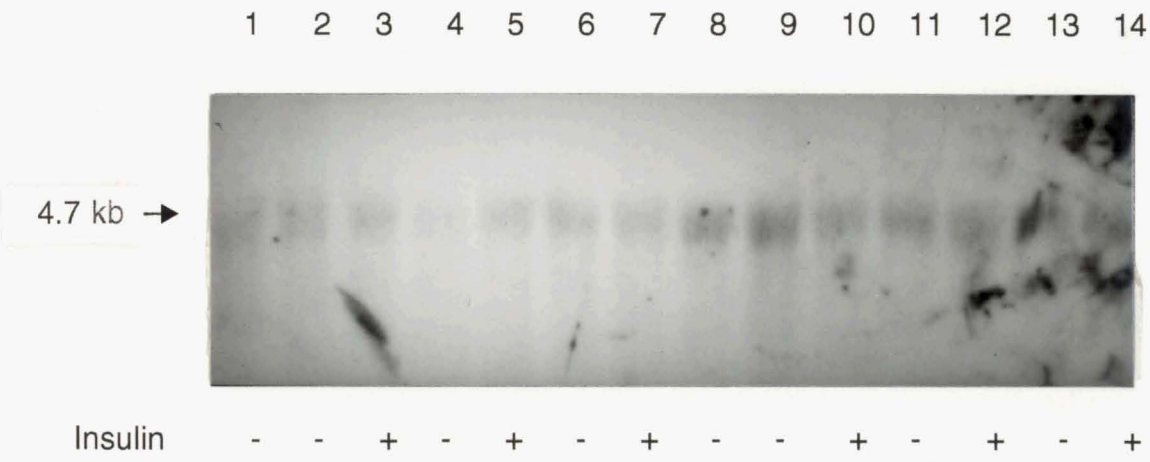


Figure 7.22: Effect of insulin infusion on insulin-like growth factor I gene expression in the wool follicle (Ullrich et al., 1986, Appendix X).

Lanes a-d) copy number controls. Lane **1)** LSS, t = 0, **2-3)** LSS, t = 30 minutes, **4-5)** LSS, t = 2 hours, **6-7)** LSS, t = 5 hours, **8)** HSS, t = 0, **9-10)** HSS, t = 30 minutes, **11-12)** HSS, t = 2 hours, **13-14)** HSS, t = 5 hours. **LSS** = low staple strength. **HSS** = high staple strength. 3 week exposure. Lanes 1-14 contain 10µg of total RNA.

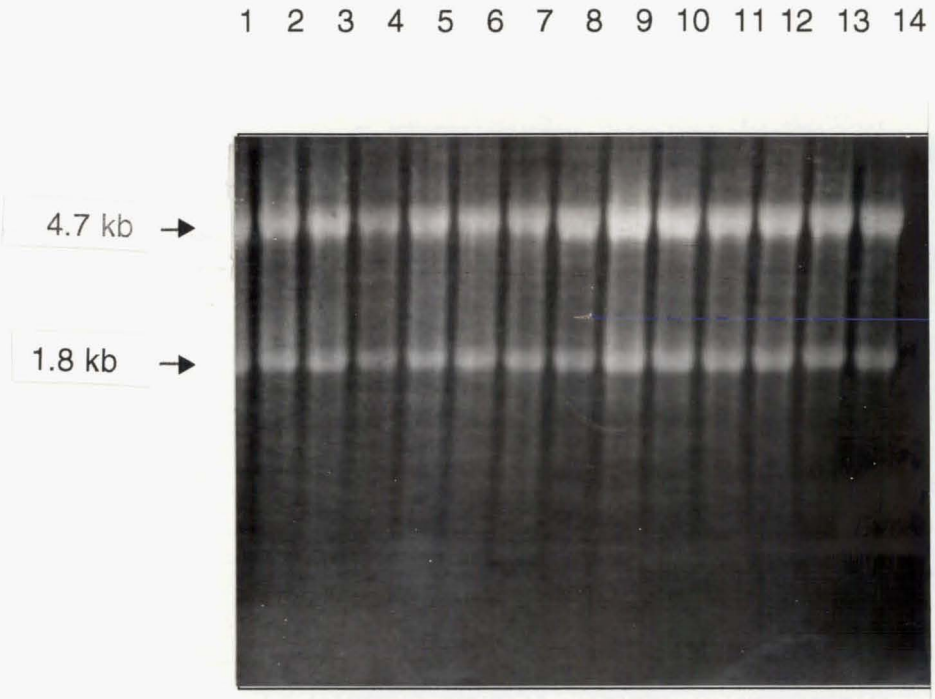
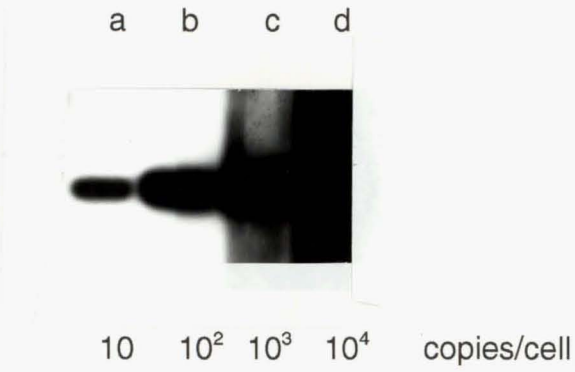


Figure 7.23: Relative transfer between samples of total RNA from the gel onto the membrane for the blot used for figure 7.24

Lanes 1-14 contain 10µg of total RNA.



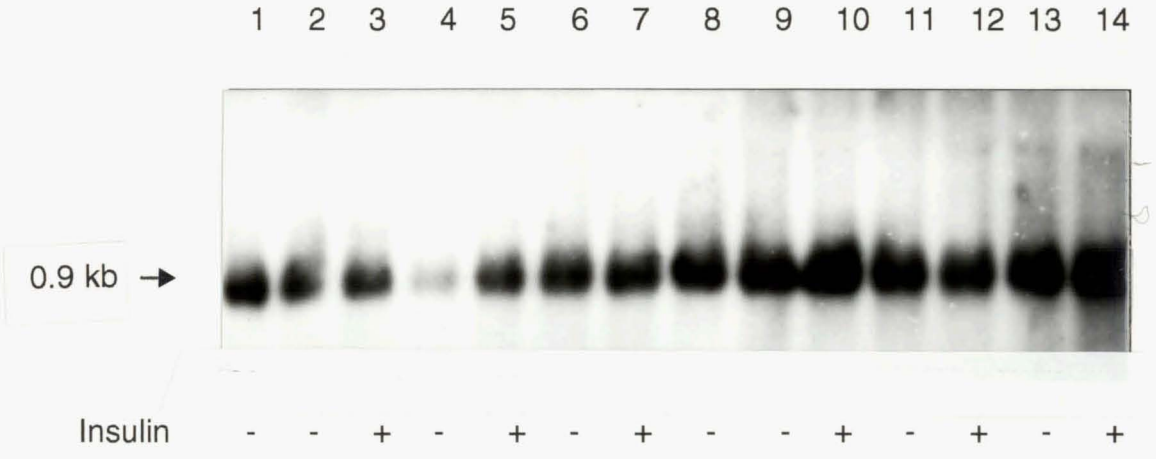


Figure 7.24: Effect of insulin infusion on ribosomal protein S6 gene expression in the wool follicle (Lott and Mackie, 1988, Appendix X).

Lanes a-d) copy number controls. Lane **1)** LSS, t = 0, **2-3)** LSS, t = 30 minutes, **4-5)** LSS, t = 2 hours, **6-7)** LSS, t = 5 hours, **8)** HSS, t = 0, **9-10)** HSS, t = 30 minutes, **11-12)** HSS, t = 2 hours, **13-14)** HSS, t = 5 hours. **LSS** = low staple strength. **HSS** = high staple strength. 3 week exposure. Lanes 1-14 contain 10µg of total RNA.

7.4 Discussion

The lack of GLUT4 expression in ovine muscle and IR and IGF-IR expression in tissues such as liver and muscle was totally unexpected since these tissues express these genes in high quantities in human and rodent models. Furthermore, the insulin receptor (Willis, 1990, Francis, 1990) and IGF-1 receptor protein at least, have been shown to reside in ovine muscle and adipose tissue, while GLUT4 protein has been found in the adipose tissue of 3-week old goats with a molecular weight similar to that reported elsewhere (Trayhun *et al.*, 1993). Why was it not possible to observe expression of these genes? Below is a discussion of some of the interpretations which can account for these results. To resolve these issues further would have required considerably more laboratory experimentation.

7.4.1 Efficiency of the hybridization protocol

It would appear that the hybridization protocol is effective since good Northern hybridization results were achieved with the B-actin and rpS6 cDNAs. Furthermore, Southern hybridization for all the cDNAs revealed that they could bind strongly to sheep DNA as well as consistently and strongly binding the copy controls. Nevertheless, GLUT1 and to some extent GLUT4 Southern hybridization of digested human genomic DNA gave different results from those reported elsewhere. In addition, Northern hybridization of GLUT1, GLUT4, IR and IGF-1R to ovine tissues was not successful apart from on one occasion for GLUT1 (as reported in this work), and on one occasion for GLUT4 (reported by another worker in our laboratory). This is despite choosing tissue in which protein expression is either known to occur (e.g. IR in ovine muscle and skin) or strongly suspected to occur (e.g. IGF-IR in liver and muscle, GLUT1 in brain and GLUT4 in muscle and adipose tissue).

The protocol used for the Southern hybridizations is a standard method recommended by the membrane manufacturer and has been used widely by other workers in the field. Modifications for Northern hybridization included the addition of 10 % dextran sulphate (which has the effect of concentrating the probe) and lowering the hybridization temperature to 50 ^{0}C which decreases the specificity of binding (a standard procedure when doing cross-species hybridization). Removing dextran sulphate from the reaction as well as lowering the hybridization temperature to 40 ^{0}C did not improve the results (data not shown). Washing conditions for both Northern and Southern hybridization can be

considered as non-stringent and reasonable stringent with 1-2xSSC/0.1% SDS wash solutions and 50 ^{0}C and 60 ^{0}C wash temperatures respectively.

Operator related problems were dismissed because Northern hybridization with the B-actin and rpS6 cDNAs was successful. Furthermore, another worker in the laboratory was having similar problems independently. This worker also repeated the GLUT1 and GLUT4 Southern hybridization experiment and achieved equivalent results. Given the standard, non-stringent nature of the protocols, in addition to the good hybridization achieved with the probe cDNA's to their copy (cDNA) standards, it was concluded that the lack of hybridization for the Northerns, and the unexpected hybridization patterns for some Southerns, was not a technical problem associated with the hybridization protocol.

7.4.2 Cross species hybridization

An obvious question to ask was 'is the homology between human cDNA and ovine genomic DNA and mRNA sufficient'? It was possible to summarise some homology data from the literature and the Genebank data base (Table 7.8) in an attempt to answer this question.

GLUT1 sequences cloned from rat, mouse and rabbit share 97-98% identity with the human GLUT1 sequence (Thorens, 1993). In addition partial sequence analysis of the bovine 'brain' (GLUT1) transporter has indicated at least 97% homology to the rat brain glucose transporter (Boado and Pardridge, 1990). Of the species for which candidate gene information was available, bovine sequences are most likely to be similar to ovine sequences. Bovine and human GLUT1 DNA sequences share 88 % homology. Importantly, Zhao *et al.*, (1993) have shown that human GLUT1 hybridizes to a bovine 2.8 kb RNA.

For the GLUT4 gene, sequence comparison between human, rat and mouse genes have shown that they share 95-96% identity (Thorens, 1993) suggesting this gene is slightly less conserved that the GLUT1 transporter. Zhao *et al.* (1993) however still show hybridization of a human GLUT4 cDNA to bovine tissues. Interestingly, the GLUT1 cDNA cross-hybridized with the GLUT4 cDNA in the copy number controls. The % homology between these two cDNAs is approximately 45%.

The insulin receptor has been suggested as being more highly conserved through evolution than insulin itself (Muggeo *et al.*, 1979) and Ullrich *et al.*, (1986) have shown that

the human IGF-1 receptor cDNA hybridized to an 11 kb transcript in rat, mouse and human tissues.

Although it can not be ruled out that the barely detectable hybridization observed in this work is the result of a poor homology between human and ovine sequences, the information described above does not provide strong evidence for this hypothesis.

---

human GLUT4 vs rat GLUT4 : 79%
Human GLUT1 vs bovine GLUT1 : 88%
Bovine IGF-1 receptor vs human IGF-1 receptor: 90%
Rat IGF-1 receptor vs human IGF-1 receptor: 89.9 %
Human IR vs rat IR: 78%
Human rpS6 vs Rat rpS6: 84.8%

Table 7.8: Genebank comparisons between cDNAs of candidate genes between species. Sequences were compared using Seqaid and its align sequences function (ktuple = 6) with the percentage homology calculated as number of paired matches/length of sequence aligned x 100.

7.4.3 Technique sensitivity

There is a strong case for concluding that expression of the candidate genes is extremely low or undetectable in ruminant tissues. Taylor *et al.*, (1992) reported undetectable expression of insulin receptor mRNA in buffalo liver cells. Also fibroblasts (skin) have been known to express low levels of insulin receptor in human studies (Rechter and Podskalny, 1976) making it likely they are expressed at even lower levels in ruminant skin.

Indeed, other workers have reported similar problems. Lobley (1994, personal communication) comments that in their work with GLUT4 expression in ovine tissues

using a human probe, expression was only observed at very low levels. This was thought to be the result of any combination of the following: 1) generally low levels of mRNA and protein synthesis in ovine tissues 2) a relative greater ease of mRNA degradation within the total RNA fraction thereby reducing the mRNA/total RNA fraction even further and 3) poor homology between human and ovine sequences. Bennet (personal communication, 1994) has also reported very low expression using human glucose transporter cDNAs in ovine tissues.

Given that copy number controls for the Northern hybridization gave good signal at the lower range (i.e. 1-10 pg for Northern hybridization) it would appear that the techniques applied were detecting low quantities of cDNA at least. Comparatively, Cheema *et al.*, 1991 reported the detection of 1-10 pg using slot blots as a very sensitive technique. It is possible that lower expression may have been detected with the use of different techniques such as a solution hybridization assay. This was beyond the scope of the techniques and expertise available within the laboratory however. Future work should certainly considered this as an option.

Final discussion and conclusions: Insulin status as a marker of wool strength in Romney sheep

The screening of high and low wool strength sheep for physiological and biochemical traits suggested that high wool strength animals had higher plasma insulin. It was unlikely that plasma insulin reflected individual variation in appetite since dietary intake had been fixed according to live weight. Others have shown that genetic differences in wool growth are independent of nutrient uptake from the digestive system (e.g. Lush, 1992). Consequently in this study, differences between high and low wool strength were assumed to be due to variation in partitioning of nutrients between wool follicles and other tissues, possibly in response to insulin. A working hypothesis was established which states that because of the increase in plasma insulin levels, high wool strength animals showed an increase in wool follicle glucose uptake. This allowed greater rates of wool protein synthesis and therefore the development of greater fibre diameters. Evidence supporting this hypothesis consisted of a) lower plasma glucose (presumably as it was removed from the blood stream by higher plasma insulin levels) b) a decrease in plasma urea (as amino acids were sequestered by the wool fibre - a tissue not involved in protein turnover) c) some peripheral tissue insulin resistance (as the result of the higher plasma insulin) and d) greater fibre diameter and wool strength.

In an attempt to provide greater understanding of the mechanisms involved in modulating glucose uptake into the wool follicle, insulin receptor binding of ovine skin, and genes expressed in the wool follicle potentially involved in glucose uptake were investigated. No differences in receptor binding between the skin of high and low wool strength animals were found. It was concluded that either post-receptor differences (at the wool follicle level) or differences in nutrient partitioning to the wool follicle were responsible for the differences in wool strength observed. While the latter certainly suggests that insulin binding and post-receptor mechanisms of muscle and adipose tissue should also be investigated, further studies centred on the former, that is post-receptor differences at the wool follicle level.

Expression of the genes for glucose transporters 1 and 4, the insulin receptor and the insulin-like growth factor I receptor, were assessed in skin, wool follicles, adipose, brain, muscle and liver tissue. The results were anticipated to characterise the 'glucose uptake apparatus' in various ovine tissues and to allow identification of their mechanisms of glucose uptake. In

addition, a major gene involved in the initiation of protein translation, a protein regulated extensively by insulin (ribosomal protein S6) was included in the study as a potential marker of protein synthesis.

Technical difficulties were encountered with northern hybridization of ovine tissues with human cDNAs. These difficulties are discussed fully in chapter 7, but briefly, experiments to eliminate these technical difficulties concluded that the most likely reason for poor hybridization was a combination of lack of homology between human and ovine sequences and low expression of these genes in ovine tissues. Nevertheless, results indicated that glucose transporter 1 was expressed in skin and brain but not in other ovine tissues, and that the expression of this gene in wool follicles was not induced by insulin. No results were obtained for glucose transporter 4, the insulin receptor or the IGF-1 receptor. Ribosomal protein S6 was expressed in all the tissues studied and was not regulated at a transcriptional level by insulin.

Future work should aim to overcome the technical difficulties encountered in this study by developing more sensitive techniques such as a solution hybridization assay (Meier *et al.*, 1991). Furthermore, the cloning of ovine versions of the candidate genes used in this work will overcome potential problems associated with cross-species hybridization. There may be other technical problems which were not identified. This conclusion however is based on the technical methods which were currently residing within our laboratory. To further develop the methodology in order to circumvent the above difficulties would have necessitated substantially more work.

While the data obtained were less descriptive of the mechanisms involved in glucose uptake than anticipated, they provided useful data for the construction of a model by which glucose uptake modulates wool growth. Ruminant skin and wool follicles have a high requirement for glucose (although it is not absolute, Leng and Stephenson, 1965, Harris *et al.*, 1989). The high requirement for glucose by the wool follicle is also indirectly implied by the large glycogen reserves (a precursor for glucose) found in the outer root sheath (Brown-Falco, 1958, Philpott and Kealey, 1991). The model therefore proposes that the high requirement for glucose by skin requires a mechanism of glucose uptake which is different from that of muscle and adipose tissue and may be similar to that of the brain. Consequently, the mechanism of uptake is likely to be insulin independent (Hom *et al.*, 1984) via glucose transporter 1 (Froehner *et al.*, 1988, Boado and Pardridge, 1990, Klip *et al.*, 1994). This work supports the model by the northern blot which showed that the expression of glucose transporter 1 occurs in skin and brain tissue, but not in adipose and muscle tissue. Human studies using keratinocytes have also shown the occurrence of GLUT1 but not GLUT4 mRNA in skin (Kosaki et al., 1991).

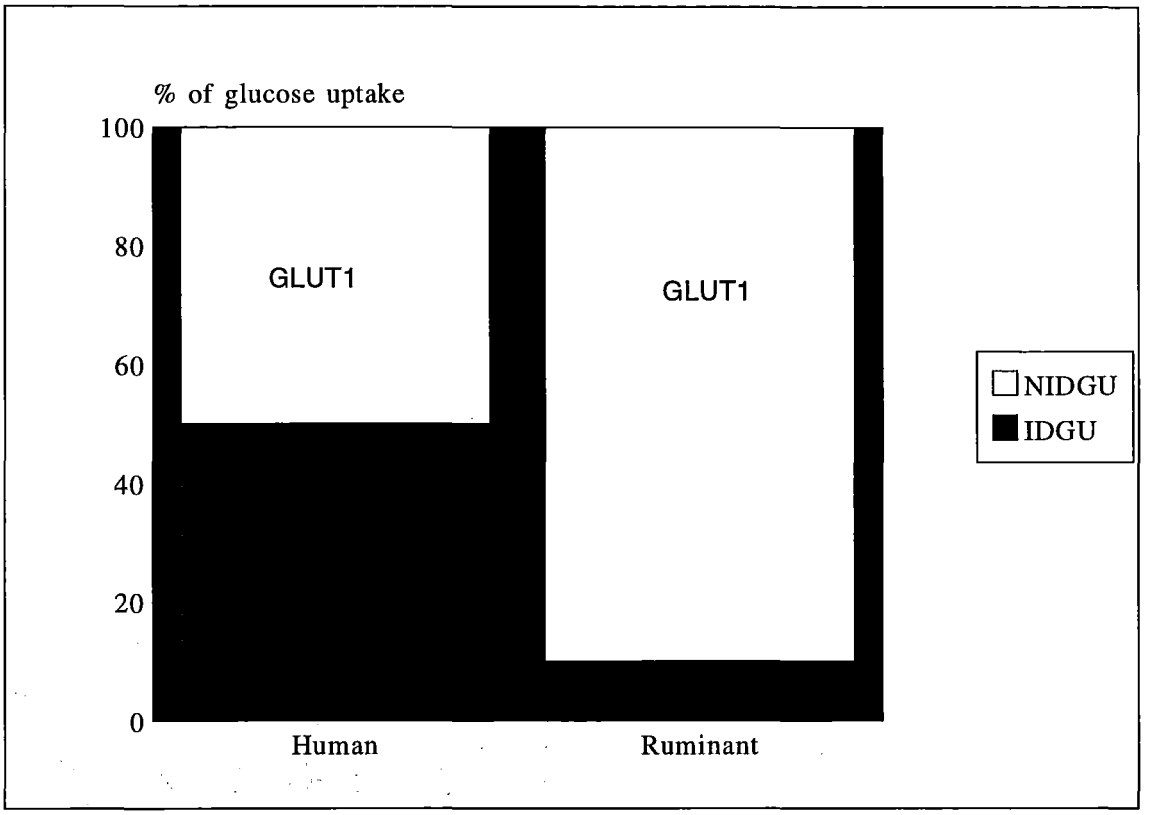


Figure 8.1: Mechanisms of glucose uptake in ruminants and in humans
Shown are the relative proportions of insulin independent (NIDGU) and insulin dependent (IDGU) glucose uptake as well as the dominant glucose transporter systems responsible for this uptake.

Skin is responsible for significant parts of whole body metabolism (Harris et al., 1989). Furthermore, 80-85 % of glucose utilization in sheep is insulin independent (Janes et al. 1985) and only 8-14% of this insulin independent glucose uptake is used by the CNS (Janes et al., 1985). This leaves a significant amount of glucose to be taken up in a non-insulin dependent manner by tissues other than the CNS. Although in primates most of the available glucose is taken up into muscle in response to insulin and via glucose transporter 4 (Koranyi *et al.*, 1991), in ruminants ketone bodies are the major fuel source for muscle tissue (Figure 8.1). Consequently, the glucose uptake in sheep is greater for skin than for resting muscle (25 versus 3-15 µmol/min/kg, Harris and Lobley 1991) and acetate uptake into the resting hind limb exceeds the net uptake into skin by a factor of two (16-25 versus 8 µmol/min/kg, Jarrett et al. 1976). Given that the ruminant skin has the potential to be a large glucose sink it is conceivable therefore, that an increase in peripheral tissue insulin resistance of insulin responsive tissue such as muscle and adipose could partition glucose to the skin where it is taken up in an insulin independent manner. Importantly, the higher plasma insulin observed in high wool strength sheep was associated with a mild increase in insulin resistance and

those tissues which traditionally show signs of insulin resistance are muscle and adipose tissue (Moller and Flier, 1991). Evidence from human studies have shown that the mechanism by which hyperinsulinemia causes insulin resistance is by down-regulation of the insulin receptor (Gavin *et al.*, 1974) and decreasing the amount of GLUT4 mRNA in muscle (Cusin *et al.*, 1990). A further possibility by which changes in partitioning to the wool follicle can occur between animals is via modulation of blood supply to the skin. Hyperinsulinamia has been shown to affect blood flow around the body in human studies which link insulin resistance to hypertension (DeFronzo and Ferrannini, 1991). Interestingly, Edwards Hocking and Hynd (1991) have shown that high wool producers show significant concurrent changes in blood flow through the skin.

While most of the discussion so far has focussed on an indirect mechanism by which insulin may affect wool follicle metabolism, it cannot be denied that insulin can potentially have a large direct effect on the skin. This is shown by the presence of insulin receptors in ovine skin in this work, and the human skin condition *acanthosis nigricans* where insulin resistance has a large impact on skin metabolism (Geffner and Golde, 1988). It is possible however, that as plasma insulin levels vary, the proportions of insulin and non-insulin mediated uptake of glucose into skin change.

In conclusion, this thesis has shown that elucidation of the mechanisms by which glucose is taken up into skin and other peripheral tissues warrants further investigation. As new methodology is developed to allow more efficient detection of ovine gene expression in relation to glucose metabolism, the impact of changes in glucose uptake on wool growth can be investigated further. Consequently, it is anticipated that the model proposed will provide a useful basis for future studies concerning the search for genetic markers of wool strength.

References

Accili, D., Frapier, C., Mosthaf, L., McKeon, C., Elbein, S.C., Permutt, M.A., Ramos, E., Lander, E., Ullrich, A., Taylor, S.I. (1989). A mutation in the insulin receptor gene that impairs transport of the receptor to the plasma membrane and causes insulin-resistant diabetes. *The EMBO journal,* 8, (9), 2509-17.

Adamo, M.L., Bach, M.A., Roberts, C.T., LeRoith, D. (1991). Regulation of insulin, IGF-I, and IGF-II gene expression. In *"Insulin-like growth factors: Molecular and cellular aspects"*, 271-304. LeRoith, D., ed. CRC Press, Boca Raton Ann Arbor Boston London.

Alexandrides, T. Moses, A.C., Smith, R.J. (1989). Developmental expression of receptors for insulin, insulin-like growth factor I (IGF-1), and IGF-II in rat skeletal muscle. *Endocrinology,* 124, (2), 1064-76.

Allain, D., Ravault, J.P., Panaretto, B.A., Rougeot, J. (1986). Effects of pinealectomy on photoperiodic control of hair follicle activity in the Limousine ram: possible relationships with plasma prolactin levels. *Journal of pineal research,* 3, 25-32.

Almeida, O.F.X., Lincoln, G.A. (1982). Photoperiodic regulation of reproductive activity in the ram: evidence for the involvement of circadian rhythms in melatonin and prolactin secretion. *Biological reproduction,* 27, 1062-75.

Anderson, D.M. (1974). The effect of feeding on the concentration of glucose and insulin in the portal and atrial plasma in pigs. *Journal of agricultural science, Cambridge,* 82, 29-36.

Anderson, C.M., Henry, R.R., Knudson, P.E., Olefsky, J.M., Webster, N.J. (1993). Relative expression of insulin receptor isoforms does not differ in lean, obese, and noninsulin-dependent diabetes mellitus subjects. *Journal of clinical endocrinology and metabolism,* 76, (5), 1380-2.

Anderson, S.L., Cox, D.R. (1950). The relation between strength and diameter of wool fibres. *Journal of the textile institute,* 41, T481-91.

Baldwin, D., Prince, M., Tsai, P., Johnson, C., Lotain, R., Rubenstein, A.H., Olefsky, J. (1981). Insulin binding, internalization, and receptor regulation in cultured human fibroblasts. *American journal of physiology,* 241, E251-60.

Ballard, F.J., Filsell, O.H., Jarrett, I.G. (1976). Amino acid uptake and output by the sheep hind limb. *Metabolism clinical and experimental,* 25, 415-8.

Baltensperger, K., Kozma, L.M., Cherniack, A.D., Klarlund, J.K., Chawla, A., Banerjee, U., Czech, M.P. (1993). Binding of the *Ras* activator son of sevenless to insulin receptor substrate-1 signaling complexes. *Science,* 260, 1950-2.

Bassett, J.M. (1968). The relation of fat and protein catabolic actions of cortisol to

glucose homeostasis in fasting sheep. *Metabolism, clinical and experimental,* 17, 644-52.

Beermann, D.H. (1989). Status of current strategies for growth regulation. In *"Animal growth regulation"*, 377-400. Campion, D.R., Hausman, G.J., Martin, R.J. eds, Plenum Press, New York and London.

Belchetz, P.E. (1984). Physiology of the anterior pituitary gland. In *"Management of pituitary disease"*, 38-68. Belchetz P.E., ed, London: Chapman and Hall.

Bell, G.I., Murray, J.C., Nakamura, Y., Kayano, T., Eddy, R.L., Fan, Y.S., Byers, M.G., Shows, T.B. (1989). Polymorphic human insulin-responsive glucose-transporter gene on chromosome 17p13. *Diabetes,* 38, 1072-5.

Bender, D.A. (1985). In *"Amino acid metabolism, 2nd edition"*. John Wiley and Sons.

Bickerstaffe, R. (1993). Regulation of nutrient partitioning in growth and lactation. *Australian journal of agricultural research,* 44, 523-39.

Bigham, M.L., Sumner, R.M.W., Hawker, H., Fitzgerald, J.M. (1983). Fleece tenderness - a review. *Proceedings of the New Zealand society of animal production,* 43, 73-8.

Binoux, M.S., Hardouin, S., Lassarre, C., Hossenlopp, P. (1982). Evidence for production by the liver of two IGF binding proteins with similar molecular weights but different affinities for IGF-1 and IGF-II. Their relationship with serum and cerebrospinal fluid IGF-1 binding proteins. *Journal of clinical endocrinology and metabolism,* 55, 600-2.

Bittman, E.L., Dempsey, R.J., Karsch, F.J. (1983). Pineal melatonin secretion drives the reproductive response to daylength in the ewe. *Endocrinology,* 113, 2276-93.

Black, J.L., Reis, P.J. (1979). Speculation on the control of nutrient partition between wool growth and other body functions. In *"Physiological and environmental limitations to wool growth"*, 269-94. Black, J.L., Reis, P.J. eds. University of New England publishing unit.

Black, J.L., Robards, G.E., Thomas, R. (1973). Effects of protein and energy intakes on the wool growth of Merino wethers. *Australian journal of agricultural research,* 24, 399-12.

Blaxter, K.L. (1962). In *"The energy supply of ruminants"*, Hutchinson, London.

Block, N.E., Menick, D.R., Robinson, K.A., Buse, M.G. (1991). Effect of denervation on the expression of two glucose transporter isoforms in rat hindlimb muscle. *Journal of clinical investigation,* 88, (5), 1546-52.

Blumenberg, M., Connolly, D.M., Freedberg, I.M. (1992). Regulation of keratin gene expression: The role of the nuclear receptors for retinoic acid, thyroid hormone, and vitamin D. *The journal of investigative dermatology,* 98, 4S-9S.

Boado, R.J., Pardridge, W.M. (1990). The brain-type glucose transporter mRNA is specifically expressed at the blood-brain barrier. *Biochemical and biophysical research communications,* 166, (1), 174-9.

Boden, G., Chen, X., DeSantis, R.A., Kendrick, Z. (1993). Effects of age and body fat on insulin resistance in healthy men. *Diabetes care,* 16, (5), 728-33.

Bolinder, J., Ostman, J., Arner, P. (1983). Influence of ageing on insulin receptor binding and metabolic effects of insulin on human adipose tissue. *Diabetes,* 32, 959-64.

Bottomley, G.A. (1979). Weather conditions and wool growth. In *"Physiological and environmental limitations to wool growth",* 115-25. Black, J.L., Reis, P.J. eds., University of New England publishing unit.

Boyd, R.D., Bauman, D.E. (1989). Mechanisms of action for somatotropin in growth. In *"Animal growth regulation",* 257-94. Campion, D.R., Hausman, G.J., Martin, R.J., eds. Plenum press, New York and London.

Braun-Falco, O. (1958). The histochemistry of the hair follicle. In *"The biology of hair growth",* 66-90. Montagna, W., Ellis, R.A. eds. Academic Press, Inc, New York.

Bray, A.R., Smith, M.C., Merrick, N.C. (1993). Wool growth responses of Romney rams selected for high or low wool strength to nutrient supplementation in winter. *Proceedings of the New Zealand society of animal production,* 53, 323-7.

Bray, A.R., Smith, M.C., Woods, J.L., Baird, D.B. (1992). Responses to selection for wool staple strength in Romney sheep. *Proceedings of the New Zealand society of animal production,* 52, 285-7.

Breiner, M., Weiland, M., Becker, W., Muller-Wieland, D., Streicher, R., Fabry, M., Joost, H.G. (1993). Heterogeneity of insulin receptors in rat tissues as detected with the partial agonist B29, B29' -suberoyl-insulin. *Molecular pharmacology,* 44, (2), 271-6.

Broad, A., Gillespie, J.M., Reis, P.J. (1970). The influence of sulphur-containing amino acids on the biosynthesis of high-sulphur wool proteins. *Australian journal of biological sciences,* 23, 149-64.

Brockman, R.P., Bergman, E.N., Joo, P.K., Manns, J.G. (1975). Effects of glucagon and insulin on net hepatic metabolism of glucose precursors in sheep. *American journal of physiology,* 229, 1344-50.

Brockman, R.P. (1983). Effects of insulin and glucose on the production and utilization of glucose in sheep (*Ovis aries). Comparative biochemistry and physiology,* 74A, 681-5.

Brockman, R.P. (1986). Pancreatic and adrenal hormonal regulation of metabolism. In *"Control of digestion and metabolism in ruminants",* 405-19. Milligan, L.P., Grovum, W.L., Dobson, A. eds, Prentice-Hall, Englewood Cliffs, N.J.

Brockman, R.P. (1976). Effect of glucagon and insulin on lipolysis and ketogenesis in sheep. *Canadian journal of comparative medicine,* 40, 166-70.

Brown, R.S., Wahl, R.L. (1993). Overexpression of Glut-1 glucose transporter in human breast cancer. An immunohistochemical study. *Cancer,* 72, (10), 2979-85.

Broyden, G. (1965). A class of methods for solving nonlinear simultaneous equations. *Mathematics of computation,* 19, 577-93.

Call, J.L., Mitchell Jr. G.E., Ely, D.G., Little, C.O. (1972). Amino acids, volatile fatty acids and glucose in plasma of insulin-treated sheep. *Journal of animal science,* 34, (5), 767-71.

Camps, M., Vilaro, S., Testar, X., Papacin, M., Zorzano, A. (1994). High and polarized expression of GLUT1 glucose transporters in epithelial cells from mammary gland: acute down-regulation of GLUT1 carriers by weaning. *Endocrinology,* 134,(2), 924-34.

Chapman, R.E., Panaretto, B.A., Frith, P.A. (1982). Changes in wool follicles of sheep following administration of dexamethasone trimethylacetate. *Journal of cell science,* 53, 323-4.

Chapman, R.E., Black, J.L. (1981). Abnormal wool growth and alopecia of artificially reared lambs. *Australian journal of biological sciences,* 34, 11-26.

Chapman, R.E., Bassett, J.M. (1970). The effects of prolonged administration of cortisol on the skin of sheep on different planes of nutrition. *Journal of endocrinology,* 48, 649-63.

Chapman, R.E. (1965). Crimp in wool: cortical segmentation and tensile properties of well-crimped and abnormally crimped fibres of Merino wool. *Australian journal of biological sciences,* 18, 689-97.

Cheema, M.A., Schumacher, H.R., Hudson, A.P. (1991). RNA-directed molecular hybridization screening: Evidence for inapparent Chlamydial infection. *The American journal of medical sciences,* 302, 261-8.

Chin, J.E., Dickens, M., Tavare, J.M., Roth, R.A. (1993). Overexpression of protein kinase C isoenzymes, α, βI, τ, and Σ in cells overexpressing the insulin receptor. Effects on receptor phosphorylation and signaling. *The journal of biological chemistry,* 268, (9), 6338-47.

Chomczynski, P., Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical biochemistry,* 162, 156-9.

Clemmons, D.R. (1991). Insulin-like growth factor binding proteins. In *"Insulin-like growth factors: Molecular and cellular aspects",* 151-80. LeRoith, D., ed, CRC Press, Boca Raton Ann Arbor Boston London.

Cocozza, S., Porcellini, A., Riccardi, G., Monticelli, A., Condorelli, G., Ferrara, A., Pianese, L., Miele, C., Capaldo, B., Beguinot, F., Varrone, S. (1992). NIDDM associated with mutation in tyrosine kinase domain of insulin receptor gene. *Diabetes,* 41, 521-6.

Collins, J.D., Chaikin, M. (1971). The mechanical properties of wool fibres and the influence of fibre structural and dimensional variation. Part I: The stress strain behaviour at various humidities. *Journal of the textile institute,* 62, 289-303.

Colly, P.A., Lange, L.A., De Ruiter, A., Van Tonder, E.M. Vermeulen, S.O., Kellerman, G.E., Whitehead, C.J. (1990). Bolo disease: a specific, localised skin disease of woolled sheep. *Journal of the South African veterinary association,* 61,(3), 90-5.

Conti, M., Jin, S.L., Monaco, L., Repaske, D.R., Swinnen, J.V. (1991). Hormonal regulation of cyclic nucleotide phosphodiesterases. *Endocrine reviews,* 12, (3), 218-34.

Corbett, J.L. (1979). Variation in wool growth with physiological state. In *"Environmental and physiological limitations to wool growth"*, 79-98. Black, J.L., Reis, P.J. eds. University of New England Publishing Unit.

Cottam, Y.H., Blair, H.T., Gallaher, B.W., Purchas, R.W., Breier, B.H. (1992). Body growth, carcass composition, and endocrine changes in lambs chronically treated with recombinantly derived insulin-like growth factor-I. *Endocrinology,* 130, (5), 2924-30.

Cuatrecasas, P. (1972). In *"Insulin action"*, 137-70. Fritz, I.B., ed, Academic Press, New York.

Cusin, I., Terrettaz, J., Rohner-Jeanrenaud, F., Zarjevski, N., Assimacopoulos-Jeannet T., Jeanrenaud, B. (1990). Hyperinsulinemia increases the amount of GLUT4 mRNA in white adipose tissue and decreases that of muscles: a clue for increased fat depot and insulin resistance. *Endocrinology,* 127 (6), 3246-9.

Cunningham, A., Lambert, G.A., Hales, J.R.S. (1979). Wool growth and adrenergic mechanisms in skin. *Proceedings of the Australian physiological and pharmacological society,* 10, 1-15.

D'Ercole, A.J., Applewhite, G.T., Underwood, L.E. (1980). Evidence that somatomedin is synthesised by multiple tissues in the fetus. *Developmental biology,* 75, 315.

Damm, P., Handberg, A., Kuhl, C., Beck-Nielsen, H., Molsted-Pederson, L. (1993). Insulin receptor binding and tyrosine kinase activity in skeletal muscle from normal pregnant women and women with gestational diabetes. *Obstetrics and gynecology,* 82, (2), 251-9.

Davis, S.R., Barry, T.N., Hughson, G.A. (1981). Protein synthesis in tissues of growing lambs. *British journal of nutrition,* 46, 409-19.

De Meyts, P., Bianco, A.R., Roth, J. (1976). Site-site interactions among insulin receptors: characterization of the negative cooperativity. *Journal of biological chemistry,* 251, 1877-88.

De Meyts, P., Roth, J. (1975). Cooperativity in ligand binding: a new graphic analysis. *Biochemical and biophysical communications,* 66, (4), 1118-26.

DeFronzo, R.A., Kraemer, N. (1981). Physical training reverses the insulin resistance of obesity. *Diabetes, supplement I,* 30, 10A.

DeFronzo, R.A., Ferrannini, E. (1991). Insulin resistance. a multifaceted syndrome

responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes care,* 14, 173-94.

Donald, A.D. (1979). Effect of parasites and disease on wool growth. In *"Physiological and environmental limitations to wool growth"*, 99-114. Black, J.L., Reis, P.J. eds, University of New England publishing unit.

Doney, J.M., Griffiths, J.G. (1967). Wool growth regulation by local skin cooling. *Animal production,* 9, 393.

Downes, A.M., Wallace, A.L.C. (1965). In *"Biology of the skin and hair growth"*, p 679.

Downes, A.M., Hutchinson, J.C.D. (1969). Effect of low skin surface temperature on wool growth. *Journal of agricultural science, Cambridge,* 72, 155-8.

Dwyer, K.J., Pardridge, W.M. (1993). Developmental modulation of blood-brain barrier and choroid plexus GLUT1 glucose transporter messenger ribonucleic acid and immunoreactive protein in rabbits. *Endocrinology,* 132, (2), 558-65.

Ebeling, P., Bourey, R., Koranyi, L., Tuominen, J.A., Groop, L.C., Henriksson, J., Mueckler, M., Soviharvi, A., Koivisto, V.A. (1993). Mechanisms of enhanced insulin sensitivity in athletes. Increased blood flow, muscle glucose transport protein (GLUT4) concentrations, and glycogen synthase activity. Journal of clinical investigations, 92, (4), 1623-31.

Ebina, Y., Ellis, L, Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J. H., Masiarz, F., Kan, Y.W., Rutter, W.J. (1985). The human insulin receptor cDNA: The structural basis for hormone-activated transmembrane signalling. *Cell,* 40, 747-58.

Edelman, I.S. (1974). Thyroid thermogenesis. *New England journal of medicine,* 290, 1303-8.

Egan, S.E. Giddings, B.W., Brooks, M.W., Buday, L., Sizeland, A.M., Weinberg, R.A. (1993). Association of *Sos Ras* exchange protein with *Grb2* is implicated in tyrosine kinase signal transduction and transformation. *Nature,* 363, 45-51.

Elsas, L.J., Longo, N. (1992). Glucose transporters. *Annual reviews in medicine,* 43, 377-93.

Eriksson, J., Lonnroth, P., Smith, U. (1992). Insulin can rapidly increase cell surface insulin binding capacity in rat adipocytes. A novel mechanism related to insulin sensitivity. *Diabetes,* 41, 707-14.

Ezaki, O., Higuchi, M., Nakatsuka, H., Kawanaka, K., Itakura, H. (1992). Exercise training increases glucose transporter content in skeletal muscles more efficiently from aged obese rats than young lean rats. *Diabetes,* 41, 920-6.

Feldman, H.A. (1983). Statistical limits in Scatchard analysis. *The journal of biological chemistry,* 258, (21), 12865-67.

Ferguson, K.A. (1949). The effect of sympathectomy on wool growth. *Australian journal of scientific research,* B2, 438-43.

Feughelman, M. (1982). The physical properties of alpha-keratin fibres. *Journal of the society of cosmetic chemists,* 33, 385-406.

Fitzgerald, J.M., Smeaton, D.C., Jagush, K.T. (1984). Fleece tenderness: the effect of nutrition, age and lambing status on pregnant Romney and Coopworth mixed-age ewes. *Proceedings of the New Zealand society of animal production,* 44, 53-6.

Flier, J.S., Usher, P., Moses, A.C. (1986). Monoclonal antibody to the type I insulin-like growth factor (IGF-1) receptor blocks IGF-1 receptor-mediated DNA synthesis: clarification of the mitogenic mechanisms of IGF-1 and insulin in human skin fibroblasts. *Proceedings of the national academy of sciences, USA,* 83, 664-8.

Flores-Riveros, J.R., McLenithan, J.C., Ezaki, O., Lane, M.D. (1993). Insulin down-regulates expression of the insulin-responsive glucose transporter (GLUT4) gene: Effects on transcription and mRNA turnover. *Proceedings of the national academy of sciences, USA,* 90, 512-6.

Foldes, A., Maxwell, C.A., Rintoul, A.J., Scaramuzzi, R.J., Baker, P. (1990). Effects of melatonin implants on selected hormonal profiles and wool growth in Merino wethers. *Proceedings of the Australian society of animal production,* 18, 208-11.

Francis, S.M. (1990). Genetic differences in glucose tolerance of sheep: Effect on insulin status and carcass composition. *Ph D thesis, Lincoln University, Canterbury, New Zealand.*

Fraser, R.D.B., MacRae, T.P., Rogers, G.E. (1972). *Keratins.* Charles C. Thomas, Springfield, Illinois.

Fraser, S., Cowen, P., Franklin, M., Franey, C., Arendt, J. (1983). Direct radioimmunoassay for melatonin in plasma. *Clinical chemistry,* 29, (2), 396-7.

Frenkel, M.J., Gillespie, J.M., Reis, P.J. (1974). Factors influencing the biosynthesis of the tyrosine-rich proteins of wool. *Australian journal of biological sciences,* 27, 31-8.

Frenkel, M.J., Gillespie, J.M., Reis, P.J. (1975). Studies on the inhibition of synthesis of the tyrosine-rich proteins of wool. *Australian journal of biological sciences,* 28, 331-8.

Frittitta, L., Vigneri, R., Papa, V., Goldfine, I.D., Grasso, G., Trischitta, V. (1993). Structural and functional studies of insulin receptors in human breast cancer. *Breast cancer research and treatment,* 25, (1), 73-82.

Froehner, S.C., Davies, A., Baldwin, S.A., Lienhard, G.E. (1988). The blood-nerve barrier is rich in glucose transporter. *Journal of neurocytology,* 17, 173-8.

Froesch, E.R., Schmid, C., Zangger, I., Schoenle, E., Eigenmann, E., Zapf, J. (1986). Effects of IGF/somatomedins on growth and differentiation of muscle and bone. *Journal of animal science, supplement 2,* 63, 57.

Fuchs, E. (1988). Keratins as biochemical markers of epithelial differentiation. *Trends in genetics,* 4, (10), 277-81.

Fukumoto, H., Kayano, T., Buse, J.B., Edwards, Y., Pilch, P.F., Bell, G.I., Seino, S. (1989). Cloning and characterization of the major insulin-responsive glucose transporter expressed in human skeletal muscle and other insulin-responsive tissues. *Journal of biological chemistry, 264,* 7776-9.

Fuller, M.F., Weekes, T.E.C., Cadenhead, A., Bruce, J.B. (1977). The protein sparing effect of carbohydrate 2. The role of insulin. *British journal of nutrition,* 38, 489-96.

Gahagan, J.H., Martin, R.J., Leach, R.M. (1980). Serum somatomedin activity in two animal models as measured using chick epiphyseal plate cartilage bioassay. *Proceedings of the society of experimental biology and medicine,* 163, 455-60.

Gammeltoft, S. (1984). Insulin receptors: binding kinetics and structure-function relationship of insulin. *Physiological reviews,* 64, (4), 1321-78.

Garrick, D.J. (1991). Best linear unbiased prediction for across-flock/year breeding values. *Proceedings of the New Zealand society of animal production,* 51, 411-6.

Gavin, J.R.III, Roth, J., Neville, D.M. Jr, DeMeyts, P, Buell, D.N. (1974). Insulin-dependent regulation of insulin receptor concentrations: A direct demonstration in cell culture. *Proceedings of the national academy of sciences, USA,* 71, 84-8.

Gee, E. (1978). Staple strength as a measure of soundness of wool: a preliminary note. *SAWTRI technical report,* no 429.

Geenty, K.G., Orwin, D.F.G., Woods, J.L., Young, S.R., Smith, M.C. (1984). Seasonal wool production and staple strength of Romney ewes. *Proceedings of the New Zealand society of animal production,* 44, 57-9.

Geffner, M.E., Golde, D.W. (1988). Selective insulin action on skin, ovary, and heart in insulin-resistant states. *Diabetes care,* 11, (6), 500-5.

Gillespie, J.M., Reis, P.J., Schinckel, P.G. (1964). The isolation and properties of some soluble proteins from wool. *Australian journal of biological sciences,* 17, 548-60.

Gillespie, J.M., Marshall, R.C., Moore, P.M., Panaretto, B.A., Robertson, D.M. (1982). Changes in the proteins of wool following treatment of sheep with epidermal growth factor. *The journal of investigative dermatology,* 79, 197-200.

Gillespie, J.M., Darskus, R.L. (1971). Relation between the tyrosine content of various wools and their content of a class of proteins rich in tyrosine and glycine. *Australian journal of biological sciences,* 24, 1189-97.

Gillespie, J.M., Frenkel, J.M. (1974). The diversity of keratins. *Comparative biochemistry and physiology,* 47B, 339-46.

Gillespie, J.M., Reis, P.J. (1966). The dietary-regulated biosynthesis of high-sulphur wool proteins. *Biochemical journal,* 98, 669-77.

Gillespie, J.M., Broad, A., Reis, P.J. (1969). A further study on the dietary-regulated biosynthesis of high-sulphur wool proteins. *Biochemical journal,* 112, 41-9.

Gliemann, J., Foley, J.E., Sonne, O., Laursen, A.L. (1985). Insulin receptor interactions. *Polypeptide hormone receptors,* 4, 1-38.

Gliemann, J., Gammeltoft, S., Vinten, J. (1975). Time course of insulin-receptor binding and insulin-induced lipogenesis in isolated rat fat cells. *Journal of biological chemistry,* 250, 3368-74.

Gluckman, P.D., Uthne, K., Styne, D.M., Kaplan, S.L., Rudolph, A.M., Grumbach, M.M. (1979). Hormone ontogeny in the ovine fetus IV. Serum somatomedin activity in the fetal and neonatal lamb and pregnant ewe: correlation with maternal and fetal growth hormone, prolactin, and chorionic somatomammotropin. *Pediatric research,* 14, 194.

Godfredson, J.A., Wheaton, J.E., Crooker, B.A., Wong, E.A., Campbell, R.M., Mowles, T.F. (1990). Growth performance and carcass composition of lambs infused for 28 days with a growth hormone-releasing factor analogue. *Journal of animal science,* 68, (11), 3624-32.

Goldfine, I.D., Vigneri, R., Cohen, D., Pliam, N.B., Kahn, C.R. (1977). Intracellular binding sites for insulin are immunologically distinct from those on the plasma membrane. *Nature,* 269, 698-700.

Gould, G.W., Holman, G.D. (1993). The glucose transporter family: structure, function and tissue-specific expression. *The biochemical journal,* 295, 329-41.

Gourdie, R.G., Orwin, D.F.G., Ranford, S., Ross, D.A. (1992). Wool fibre tenacity and its relationship to staple strength. *Australian journal of agricultural research,* 43, 1759-76.

Gourdie, R. (1989). The relationship between wool fibre intrinsic material strength variation and staple strength in a flock of New Zealand Romney ewes. *PhD thesis, Lincoln College.*

Grigorescu, F. (1988). Analysis of insulin binding data: Clinical applications. In *"Insulin receptors, Part B: Clinical assessment, biological responses, and comparison to the IGF-1 receptor",* 121-34. Kahn, C.R., Harrison, L.C., eds, Alan R. Liss, Inc., New York.

Guesnet, M., Massoud, M.J., Demame, Y. (1991). Regulation of adipose tissue metabolism during pregnancy and lactation in the ewe: The role of insulin. *Journal of animal science,* 69, 2057-65.

Guler, H.P., Binz, K., Eigenmann, E., Jaggi, S., Zimmermann, D., Zapf, J., Froesch, E.R. (1989). Small stature and insulin-like growth factors: prolonged treatment of mini-poodles with recombinant human insulin-like growth factor I. *Acta endocrinologia,* 121, 456-64.

Guma, A., Vinals, F., Camps, M., Lizarbe, M., Mora, C., Bertran, J., Testar, X., Palacin, M., Zorzano, A. (1993). Effect of benzyl succinate on insulin receptor function and insulin action in skeletal muscle: further evidence for a lack of spare high-affinity insulin receptors. *Molecular and cellular*

endocrinology, 91, 29-33.

Gunning P., Ponte, P., Okayama, H., Engel, J., Blau, H., Kedes, L. (1983). Isolation and characterization of full-length cDNA clones for human α- β- and τ- Actin mRNAs: Skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. *Molecular and cellular biology*, 3, 787-95.

Hadley, M.E. (1988). Thyroid Hormones. In *"Endocrinology, 2nd edition"*, 299-324. Prentice Hall, Englewood Cliffs, New Jersey.

Hansford, K.A. (1992). Fibre diameter distribution: Implications for wool production. *Wool technology and sheep breeding*, 40, (1), 2-9.

Hansford, K.A., Kennedy, J.P. (1988). Relationship between the rate of change in fibre diameter and staple strength. *Proceedings of the Australian society of animal production*, 17, 415.

Hansford, K.A., Emery, G.B., Teasdale, D.C. (1985). A method to measure the diameter profile of wool staples. *Wool technology and sheep breeding*, 33, (1), 25-7.

Haring, H.U. (1991). The insulin receptor: Signalling mechanism and contribution to the pathogenesis of insulin resistance. *Diabetologia*, 34, 848-61.

Haring, H., Kirsch, D., Obermaier, B., Ermel, B., Machicao, F. (1986). Decreased tyrosine kinase activity of insulin receptor isolated from rat adipocytes rendered insulin-resistant by catecholamine treatment in vitro. *Biochemical journal*, 234, 59.

Harris, P.M., Dellow, D.W., Sinclair, B.R. (1988). Measurement of blood flow and amino acid metabolism in the skin of sheep. *Proceedings of the New Zealand society of animal production*, 48, 187-9.

Harris, P.M., Xu, Z.Z., Blair, H.T., Dellow, D.W., McCutcheon, S.N., Cockrem, J. (1989). The effect of exogenous melatonin, administered in summer, on wool growth and testis diameter of Romneys. *Proceedings of the New Zealand society of animal production*, 49, 35-8.

Harris, P.M., Lobley, G.E. (1991). Amino acid and energy metabolism in the peripheral tissues of ruminants. In "Physiological aspects of digestion and metabolism in ruminants. *Proceedings of the seventh international symposium on ruminant physiology"*, 201-30. Tsuda, T., Sasaki, Y., Kawashima, R. eds. Academic Press, Inc.

Harris, P.M., Dellow, D.W., Sinclair, B.R. (1989). Preliminary *'in vivo'* measurements of protein and energy metabolism in the skin of sheep. *Australian journal of agricultural research*, 40, 879-88.

Hart, D.S. (1961). The effect of light-dark sequences on wool growth. *Journal of agricultural science*, 56, 235-42.

Hart, D.S., Bennett, J.W., Hutchinson, J.C.D., Wodzicka-Tomaszewska, M. (1963). Reversed photoperiodic seasons and wool growth. *Nature*, 198, 310-11.

Hawker, H. (1984). The effect of seasonality and time of shearing on staple strength. In *"Staple strength. Report of a seminar on staple strength held at Lincoln College, August, 1984. Lincoln College Wool Bulletin"*, 1, 43-8.

Hawker, H., Dodds, K.G., Andrews, R.N., McEwan, J.C. (1988). Production and characteristics of wool from the hogget progeny of sheep intensively screened for fleece weight. *Proceedings of the New Zealand society of animal production*, 48, 207-12.

Hawker, H., Crosbie, S.F. (1985). Effects of level of nutrition in winter and summer on the wool growth of Romney and Perendale ewes with a history of high or low winter wool growth. *Proceedings of the New Zealand society of animal production*, 45, 225-8.

Hawker, H., Littlejohn, R.P. (1989). Relationships between staple strength and other wool characteristics of Romney ewes. *New Zealand journal of agricultural research*, 32, 305-10.

Hayashi, H., Nishioka, Y., Kamohara, S., Kanai, F., Ishii, K., Fukui, Y., Shibasaki, F., Takenawa, T., Kido, H., Katsunuma, N. ***et al*** (1993). The alpha-type 85-kDa subunit of phosphatidylinositol 3-kinase is phosphorylated at tyrosines 368, 150, and 607 by the insulin receptor. *Journal of biological chemistry*, 268, (10), 7107-17.

Hecht, L.B., Straus, D.S. (1990). Insulin-sensitive, serum-sensitive protein kinase activity that phosphorylates ribosomal protein S6 in cultured fibroblast-melanoma hybrid cells. *Endocrinology*, 119, 470-80.

Heidenreich, K.A., Olefsky, J.M. (1985). The metabolism of insulin receptors. Internalization, degradation, and recycling. In *"Molecular basis of insulin action"*, 45-66. Czech, M.P. ed., Plenum Press: New York, London.

Heidenreich, K.A., Zahniser, N.R., Berhanu, P., Brandenburg, D., Olefsky, J.M. (1983). Structural differences between insulin receptors in the brain and peripheral target tissues. *The journal of biological chemistry*, 258, (14), 8527-30.

Hemsley, J.A., Reis, P.J. (1985). Amino acids and wool growth. In *"Ruminant physiology - concepts and consequences"*, proceedings of a symposium held at University of Western Australia, 253-61. Baker, S.K., Gawthorne, J.M., Mackintosh, J.B., Purser, D.B., eds. University of Western Australia.

Henderson, C.R., Kempthorne, O., Searle, S.R., von Krosigk, C.M. (1959). The estimation of environmental and genetic trends from records subject to culling. *Biometrics*, 15, 192.

Heuer, P.A. (1979). Staple strength - measurement equipment. In *"Proceedings of the seminar on staple length and staple strength of greasy wool. CSIRO division of textile physics"*, 63-78.

Hewish, D.R., French, P.W. (1986). Monoclonal antibodies to a subfraction of Merino wool high-tyrosine proteins. *Australian journal of biological sciences*, 39, 341-51.

Hickford, J.G.H. (1989). The HLA class II region of IDDM discordant, IDDM

concordant and healthy monozygotic twins: Evidence of somatic recombination. *Ph D thesis, University of Otago, Dunedin, New Zealand.*

Hintz, R.L., Liu, F., Rosenfeld, R.G., Kemp, S.F. (1981). Plasma somatomedin-binding proteins in hypopituitarism: Changes during growth hormone therapy. *Journal of clinical endocrinology and metabolism,* 53, 100.

Hocking Edwards, J.E., Hynd, P.I. (1991). Blood flow through the skin of high and low wool producers. *Proceedings of the nutrition society of Australia,* 16, 206.

Hodgkinson, S.S., Moore, L.G., Napier, F.R., Dans, S.R., Bass, J.J., Giuckman, P.D., (1989). Characterisation of insulin-like growth factor binding proteins in ovine tissue fluids. *Journal of Endocrinology,* 120, 429-438.

Hodgkinson, S.S., Bass, J.J., Gluckman, P.D. (1991). Plasma IGF-1 binding proteins in sheep: Effect of recombinant growth hormone treatment and nutritional status. *Domestic Animal Endocrinology,* 8(3), 343-351.

Hoey, W.A., Hopkins, P.S. (1983). Chronic arterial cannulation for studying the skin of sheep. *Research in veterinary science, 35,* 247-9.

Hollenburg, M.D. (1985). Insulin, its receptor and diabetes: An overview. In "*Insulin, its receptor and diabetes.*" Hollenburg, M.D.

Hom, F.G., Goodner, C.J., Berrie, M.A. (1984). A (3H)-2deoxyglucose method for comparing rates of glucose metabolism and insulin responses among rat tissues *in vivo.* Validation of the model and the absence of an insulin effect on the brain. *Diabetes,* 33, 141-52.

Houghton, D.C., Walker, D.W., Young, I.R., McMillen, I.C. (1993). Melatonin and light-dark cycle separately influence daily behavioral and hormonal rhythms in the pregnant ewe and sheep fetus. *Endocrinology,* 133, (1), 90-8.

Hunter, L, Leeuwner, W., Smuts, S., Strydom, M.A. (1983). The correlation between staple strength and single fibre strength for sound and tender wools. *SAWTRI technical report,* 514, 15 pp.

Hynd, P.I. (1989). Effects of nutrition on wool follicle cell kinetics in sheep differing in efficiency of wool production. *Australian journal of agricultural research,* 40, 409-17.

Hynd, P.I. (1989). Factors influencing cellular events in the wool follicle. In *"The Biology of wool and hair",* 169-84. Rogers, G.E., Reis, P.J., Ward, K. A., Marshall, R.C. eds, Chapman and Hall.

Ingbar, D.H., Galton, V.A. (1975). The effect of food deprivation on the peripheral metabolism of thyroxine in rats. *Endocrinology,* 96, 1525-1532.

Jacob, R., Barett. E., Plewe, G., Fagin, K.D., Sherwin, K.S. (1989). Acute effects of insulin-like growth factor 1 on glucose and amino acid metabolism in the awake fasted rat. *Journal of Clinical Investigation,* 83, 1717.

Jacobs, S., Shechter, Y., Bissell, K., Cuatrecasas, P. (1977). Purification and properties of insulin receptors from rat liver membranes. *Biochemical and biophysical*

research communications, 77, (3), 981-8.

Janes, A.N., Weekes, T.E.C., Armstrong, D.G. (1985). Insulin action and glucose metabolism in sheep fed on dried-grass or ground, maize-based diets. *British journal of nutrition*, 54, 459-71.

Jarrett, I.G., Filsell, O.H., Ballard, F.J. (1974). Metabolic and endocrine interrelationships in normal and diabetic sheep. In *"Lipid metabolism, obesity and diabetes mellitus: impact upon artherosclerosis"*. Hormone and metabolic research, international symposium, 111-6. Greten, H., Levine, R., Pfeiffer, E.F., Renold, A.E. eds., Georg Thieme Publishers, Stuttgart.

Jarrett, I.G., Filsell, O.H., Ballard, F.J. (1976). Utilization of oxidizable substrates by the sheep hind limb: effects of starvation and exercise. *Metabolism*, 25, 523-31.

Jarvis, T.M., Hynd, P.I., Snoswell, A.M. (1990). Activity of polyamine-synthesising enzymes in wool follicles. *Proceedings of the Australian society of animal production*, 18, 498.

Kahn, R. (1976). Membrane receptors for hormones and neurotransmitters. *The journal of cell biology*, 70, 261-86.

Kahn, C.R. (1978). Insulin resistance, insulin insensitivity, and insulin unresponsiveness: a necessary distinction. *Metabolism*, 27, (12), 1893-1902.

Kahn, C.R., White, M.F. (1988). The insulin receptor and the molecular mechanism of insulin action. *Journal of clinical investigations*, 82, 1151-6.

Kahn, C.R., Podskalny, J.M. (1980). Demonstration of a primary (? genetic) defect in insulin receptors in fibroblasts from a patient with the syndrome of insulin resistance and acanthosis nigricans type A. *Journal of clinical endocrinology and metabolism*, 50, 1139-41.

Kahn, C.R., Freychet, P., Roth, J. (1974). Quantitative aspects of the insulin-receptor interaction in liver plasma membranes. *Journal of biological chemistry*, 7, 2249-57.

Kahn, B.B., Flier, J.S. (1990). Regulation of glucose-transporter gene expression *in vitro* and *in vivo*. *Diabetes care*, 13, (6), 548-64.

Kaiser, N., Sasson, S., Feener, E.P., Boukobza-Vardi, N., Higashi, S., Moller, D.E., Davidheiser, S., Przybylski, R.J., King, G.L. (1993). Differential regulation of glucose transport and transporters by glucose in vascular endothelial and smooth muscle cells. *Diabetes*, 42, 80-9.

Karnieli, E., Garvey, W.T., Olefsky, J.M., Hueckstead, T.P., Harel, C., Maianu, L., Armoni, M. (1993). Potential role for insulin and cycloheximide in regulating the intrinsic activity of glucose transporters in isolated rat adipocytes. *Endocrinology*, 133 (6), 2943-50.

Kasanicki, M.A., Pilch, P.F. (1990). Regulation of glucose-transporter function. *Diabetes care*, 13, (3), 219-27.

Kennedy, P.M., Christopherson, R.J., Milligan, L.P. (1985). In *"Control of digestion and metabolism in ruminants"*, Milligan, L.P., Grovum, W.L., Dobson, A. eds. Reston, Va., U.S.: Reston publishing Co.

Kirton, A.H., Cresswell, E., Cockrem, F.R., Butler, G.W. (1959). Some effects of thyroxine on live weight, metabolism and wool growth of Romney ewes. *New Zealand journal of agricultural research,* 2, (6), 1143-58.

Klip, A., Tsakiridis, T., Marett, A., Ortiz, P.A. (1994). Regulation of expression of glucose transporters by glucose: A review of studies *in vivo* and in cell cultures. *FASEB journal,* 8, 43-53.

Klotz, I.M. (1982). Number of receptor sites from Scatchard graphs: Facts and fantasies. *Science,* 217, 1247-9.

Kohrt, W.M., Kirwan, J.P., Staten, M.A., Bourey, R.E., King, D.S., Holloszy, J.O. (1993). Insulin resistance in ageing is related to abdominal obesity. *Diabetes,* 42, 273-81.

Kono, T., Barham, F.W. (1971). The relationship between the insulin-binding capacity of fat cells and the cellular response to insulin: Studies with intact and trypsin-treated fat cells. *Journal of biological chemistry,* 246, 6210-6.

Koontz, J.W., Iwahashi, M. (1981). Insulin as a potent, specific growth factor in a rat hepatoma cell line. *Science,* 211, 947-49.

Koranyi, L.I., Bourey, R.E., Vuorinen-Markkola, H., Koivisto, V.A., Mueckler, M., Permutt, M.A., Yki-Jarvinen, H. (1991). Level of skeletal muscle glucose transporter protein correlates with insulin-stimulated whole body glucose disposal in man. *Diabetologia,* 34, (10), 763-5.

Kosaki, A., Kuzuya, H., Nishimura, H., Inoue, G., Okamoto, M., Kakehi, T., Okamoto, M., Kono, S., Maeda, I., Kubota, M., Imura, H. (1991). Regulation of glucose transporter synthesis in cultured human skin fibroblasts. *Molecular and cellular endocrinology,* 75, 189-96.

Koyanagi, T., Odagiri, S. (1960). Effect of vitamin A on the cystine content in skin or rats. *Nature,* 186, 809.

Koyanagi, T., Takanohashi, T. (1961). Cystine content in hair of children as influenced by vitamin A and animal protein in the diet. *Nature,* 192, 457-8.

Kozma, L., Baltensperger, K., Klarlund, J., Porras, A., Santos, E., Czech, M.P. (1993). The *ras* signaling pathway mimics insulin action on glucose transporter translocation. *Proceedings of the national academy of sciences of the USA,* 90, (10), 4460-4.

Krieg, J., Hofsteenge, J., Thomas, G. (1988). Identification of the 40 S ribosomal protein S6 phosphorylation sites induced by cycloheximide. *The journal of biological chemistry,* 263, 11473-77.

Lee, H.J. (1956). The influence of copper deficiency on the fleeces of British breeds of sheep. *The journal of agricultural science,* 47, 218 - 224.

Leng, R.A., Stephenson, S.K. (1965). Glucose and acetate metabolism by isolated sheep wool follicles. *Archives of biochemistry and biophysics,* 110, 8-15.

Leng, R.A., West, C.E. (1969). Contribution of acetate, butyrate, palmitate, stearate and oleate to ketone body synthesis in sheep. *Research in veterinary science,* 10, 57-63.

Lever, M. (1977). Carbohydrate determination with 4 - hydroxybenzoic acid hydrazide (PAHBAH): Effect of bismuth on the reaction. *Analytical biochemistry,* 81, 21-7.

Lewer, R.P., Rae, A.L., Wickham, G.A. (1983). Analysis of records of a Perendale flock V. Repeatabilities and genetic correlations between ages. *New Zealand journal of agricultural research,* 26, 315-9.

Lewis, J.G., Manley, L., Whitlow, J.C., Elder, P.A. (1992). Production of a monoclonal antibody to cortisol: Application to a direct enzyme-linked immunosorbent assay of plasma. *Steroids,* 57, 82-5.

Li, S.R., Baroni, M.G., Oeobaum, R.S., Stock, J., Galton, D.J. (1988). Association of genetic variant of the glucose transporter with non-insulin-dependent diabetes mellitus. *The lancet,* 368-70.

Lincoln, G.A., Libre, E.A., Memain, G.R. (1989). Long-term reproductive cycles in rams after pinealectomy or superior cervical ganglionectomy. *Journal of reproduction and fertility,* 85, (2), 687-704.

Lincoln, G.A., Klandorf, H., Anderson, N. (1980). Photoperiodic control of thyroid function and wool and horn growth in rams and the effect of cranial sympathectomy. *Endocrinology,* 107, 1543-8.

Lincoln, G.A., Almeida, O.F.X., Klandorf, H., Cunningham, R.A. (1982). Hourly fluctuations in the blood levels of melatonin, prolactin, luteinizing hormone, follicle-stimulating hormone, testosterone, tri-iodothyronine, thyroxine and cortisol in rams under artificial photoperiods and the effects of cranial sympathectomy. *Journal of endocrinology,* 92, 237-50.

Lincoln, G.A., Ebling, F.J.P. (1985). Effect of constant-release implants of melatonin on seasonal cycles in reproduction, prolactin secretion and moulting in rams. *Journal of reproduction and fertility,* 73, 241-53.

Lincoln, G.A. (1984). Central effects of photoperiod on reproduction in the ram revealed by the use of a testosterone clamp. *Journal of endocrinology,* 103, 233-41.

Linder, M.C. (1991). Nutrition and metabolism of proteins. In *"Nutritional biochemistry and metabolism with clinical application"*, 87-109. Linder, M.C. ed, Elsevier.

Lindner, H.R., Ferguson, K.A. (1956). Influence of the adrenal cortex on wool growth and its relation to 'break' and 'tenderness' of the fleece. *Nature,* 1477, 188-9.

Lindsay, D.B. (1970). In *"Physiology of digestion and metabolism in the ruminant"*, 439-51. Phillipson, A.T. ed, Newcastle upon Tyne, Oriel Press.

Lobley, G.E., Connell, A., Buchan, V. (1987). Effect of food intake on protein and energy metabolism in finishing beef steers. *British journal of nutrition,* 57, 457-65.

Lonnroth, P. (1991). Regulation of insulin action at the cellular level. *Journal of internal medicine,* 229, 23-9.

Lott, J.B., Mackie, G.A. (1988). Isolation and characterization of cloned cDNAs that code for human ribosomal protein S6. *Gene,* 65, 31-9.

Loudon, A.S.I., Milne, J.A., Curlewis, J.D., McNeilly, A.S. (1989). A comparison of the seasonal hormone changes and patterns of growth, voluntary food intake and reproduction in juvenile and adult red deer (*Cervus elaphus*) and Pere David's deer (*Elaphus davidianus*) hinds. *Journal of endocrinology,* 122, 733-45.

Lowe Jr, W.L. (1991). Biological actions of the insulin-like growth factors. In *"Insulin-like growth factors: Molecular and cellular aspects"*, 50-86. LeRoith, D. ed. CRC Press: Boca Raton Ann Arbor Boston London.

Lush, J.M. (1992). The physiological basis for differences in wool production between high and low wool producing genotypes. *PhD thesis, University of Sydney.*

Lynch, L.J., Michie, N.A. (1976). An instrument for the rapid automatic measurement of fibre-fineness distribution. *Textile research journal,* 46, 653-60.

Lyne, A.G., Jolly, M., Hollis, D.E. (1970). Effects of experimentally produced local subdermal temperature changes on skin temperature and wool growth in the sheep. *Journal of agricultural science,* 74, 83.

Maddever, D.C., Carnaby, G.A., Ross, D.A. (1991). Relative economic values of wool processing parameters. *Proceedings of the New Zealand society of animal production,* 51, 333-8.

Magnuson, M.A., Granner, D.K. (1988). Analyzing the effects of insulin on gene expression. In *"insulin receptors Part B: Clinical assessment, biological responses and comparison to the IGF-1 receptor"*, Kahn, C.R., Harrison, L.C. eds. Receptor biochemistry and methodology, 12 B, 189-210. Venier, J.C., Harrison, L.C. series eds. Alan R Liss. Inc. New York.

Mamula, P.., McDonald, A.R., Brunetti, A., Okabayashi, Y., Wong, K.Y., Maddux, B.A., Logsdon, C., Goldfine, I.D. (1990). Regulating insulin-receptor-gene expression by differentiation and hormones. *Diabetes care,* 13, (3), 288-301.

Marceau, N., Grenier, A., Noel, M., Mailhot, D., Loranger, A. (1992). Modulation of cytokeratin and actin gene expression and fibrillar organization in cultured rat hepatocytes. *Biochemical and cellular biology,* 70, 1238-48.

Marinetti, G.V., Schlatz, L., Reilly, K. (1972). In *"Insulin action"*, 207-96. Fritz, I.B., ed, Academic Press, New York.

Marshall, D.J., Walker, R.I., Cullies, B.R., Luff, M.F. (1991). The effect of footrot on body weight and wool growth of sheep. *Australian veterinary journal,* 68, (2), 45-9.

Marshall, S., Olefsky, J.M. (1980). Effects of insulin incubation on insulin binding, glucose transport, and insulin degradation by isolated rat adipocytes. Evidence for hormone-induced desensitization at the receptor and post-receptor level. *Journal of clinical investigations,* 66, 763-72.

Marshall, S. Olefsky, J.M. (1983). Separate intracellular pathways for insulin receptor recycling and insulin degradation in isolated rat adipocytes. *Journal of cell physiology,* 117, 195-203.

Marshall, R.C., Gillespie, J.M. (1989). Variations in the proteins of wool and hair. In *"The biology of wool and hair"*, 117-23. Rogers, G.E., Reis, P.J., Ward, K.A., Marhsall, R.C., eds, Chapman and Hall, London & New York.

Marston, H.R., Lee, H.J. (1948). The effect of copper deficiency and of chronic overdosage with copper on Border-Leicester and Merino sheep. *Journal of agricultural research,* 38, 229-41.

Marston, H.R. (1946). Nutrition and wool production. In *"Society of dyers and colourist. Proceedings of a symposium on fibrous proteins, Leeds, May 1946"*, 207-14.

Massague, J., Pilch, P.F., Czech, M.P. (1980). Electrophoretic resolution of three major insulin receptor structures with unique subunit stoichiometries. *Proceedings of the national academy of sciences, USA,* 77, 7137-41.

Massague, J., Pilch, P.F., Czech, M.P. (1981). A unique proteolytic cleavage site on the ß subunit of the insulin receptor. *The journal of biological chemistry,* 256, (7), 3182-3190.

Masters, D.G., Chapman, R.E., Vaughan, J.D. (1985). Effects of zinc deficiency on the wool growth, skin and wool follicles of pre-ruminant lambs. *Australian journal of biological sciences,* 38, 355-64.

Maudelonde, T., Rosenfield, R.L., Shuler, C.F., Schwartz, S.A. (1986). Studies of androgen metabolism and action in cultured hair and skin cells. *Steroid biochemistry,* 24, (5), 1053-60.

McClain, D.A. (1991). Different ligand affinities of the two human insulin receptor splice variants are reflected in parallel changes in sensitivity for insulin action. *Molecular endocrinology,* 5, 734-9.

McClelland, L.A., Blair, H.T., Wickham, G.A., Brookes, I.M. (1986). A comparison of rams from fleece weight-selected and randomly bred Romney flocks for intake, wool growth and feed utilisation. *Proceedings of the New Zealand society of animal production,* 46, 215-8.

McCutcheon, S.N., Mackenzie, D.D.S., Blair, H.T. (1987). Nitrogen metabolism and plasma urea concentrations in fleeceweight-selected and control Romney rams. *Australian journal of agricultural research,* 38, 917-26.

McDonald, J.M., Pershadsingh, (1985). The role of calcium in the transduction of insulin action. In *"Molecular basis of insulin action"*, 103-118. Czech, M.P., ed. Plenum Press: New York, London.

McDonald, B.J., Hoey, W.A., Hopkins, P.S. (1987). Cyclical fleece growth in

Moore, G.P.M., Jackson, N., Lax, J. (1989). Evidence of a unique developmental mechanism specifying both wool follicle density and fibre size in sheep selected for single skin and fleece characters. *Genetics research, Cambridge,* 53, 57-62.

Moore, L.G., Mylek, M.E. (1993). A novel method for the extraction of sheep insulin-like growth factor I and II from plasma prior to radioimmunoassay. *Journal of endocrinology,* 137, 239-45.

Moore, R.D. (1985). The case for intracellular pH in insulin action. In *"Molecular basis of insulin action"*, 145-70. Czech, M.P., ed. Plenum Press: New York, London.

Moriya, K., Ito, K., Sasaki, Y. (1985). Roles of adrenal hormones to maintain plasma glucose level after insulin injection in sheep. In *"Proceedings of the 3rd animal science congress of the Asian-Australasian association of animal production societies"*. 2, 820-22.

Moses, A.C., Tsuzaki, S. (1991). Is insulin a growth factor? In *"Insulin-like growth factors: Molecular and cellular aspects"*, 245-70. LeRoith, D., ed, CRC Press, Boca Raton Ann Arbor Boston London.

Mosthaf, L., Eriksson, J., Harin, H.U., Groop, L., Widen, E., Ullrich, A. (1993). Insulin receptor isotype correlates with risk of non-insulin-dependent diabetes. *Proceedings of the national academy of sciences, USA,* 90, 7, 2633-5.

Mueckler, M. (1990). Family of glucose-transporter genes. Implications for glucose homeostasis and diabetes. *Diabetes,* 39, 6-11.

Mueckler, M., Caruso, C., Baldwin, S.A., Panico, M., Blench, I., Morris, H.R., Allard, W.J., Lienhard, G.E., Lodish, H.F. (1985). Sequence and structure of a human glucose transporter. *Science,* 229, 941-5.

Muggeo, M., ginsberg, B.H., Rothe, J., Neville, D.M., Jr., De Meyts, P., Kahn, C.R. (1979). The insulin receptor in vertebrates is functionally more conserved than insulin itself. *Endocrinology,* 104, 1393.

Nay, T. (1966). Wool follicle arrangement and vascular pattern in the Australian Merino. *Australian journal of agricultural research,* 17, 797-805.

Nicholls, T.J., Follett, B.K., Goldsmith, A.R., Pearson, H. (1988). Possible homologies between photorefractoriness in sheep and birds: The effect of thyroidectomy on the length of the ewe's breeding season. *Reproduction, nutrition, development,* 3, 75-85.

Nicoll, C.S. (1974). Physiological actions of prolactin. In *"Handbook of physiology: endocrinology IV, part 2 "*, 253-92.

Norgren, S., Arner, P., Luthman, H. (1994). Insulin receptor ribonucleic acid levels and alternative splicing in human liver, muscle, and adipose tissue. Tissue specificity and relation to insulin action. *Journal of clinical endocrinology and metabolism,* 78, 757-62.

Norgren, S., Zierath, J., Galuska, D., Wallberg-Hendriksson, H., Luthman, H. (1993).

Differences in the ratio of RNA encoding two isoforms of the insulin receptor between control and NIDDM patients. *Diabetes*, 42, 675-81.

O' Brien, R.M., Granner, D.L. (1991). Regulation of gene expression by insulin. *The biochemical journal*, 278, 609-19.

Oddy, V.H., Gooden, J.M., Hough, G.M., Teleni, E., Annison, E.F. (1985). Partitioning of nutrients in Merino ewes. II Glucose utilization by skeletal muscle, the pregnant uterus and the lactating mammary gland in relation to whole body glucose utilization. *Australian journal of biological sciences*, 38, 95-108.

Oddy, V.H., Lindsay, D.B. (1986). Metabolic and hormonal interactions and their potential effects on growth. In *"Control and manipulation of animal growth"*, 231-48. Buttery, P.J., Haynes, N.B., Lindsay, D.B. eds, Butterworths.

Oddy, V.H., Lindsay, D.B., Barker, P.J., Northrop, A.J. (1987). Effect of insulin on hind-limb and whole-body leucine and protein metabolism in fed and fasted lambs. *British journal of nutrition*, 58, 437-52.

Oddy, V.H. (1985). Wool growth of pregnant and lactating Merino ewes. *Journal of agricultural science, Cambridge*, 105, 613-22.

Okabayashi, Y., Maddux, B.A., McDonald, A.R., Logsdon, C.D., Williams, J.A., Goldfine, I.D. (1989). Mechanisms of insulin-induced insulin-receptor downregulation. Decrease of receptor biosynthesis and mRNA levels. *Diabetes*, 38, 182-7.

Olefsky, J.M. (1976). Effects of fasting on insulin binding, glucose transport, and glucose oxidation in isolated rat adipocytes: Relationships between insulin receptors and insulin action. *Journal of clinical investigations*, 58, 1450-60.

Olefsky, J.M., Reaven, G.M. (1975). Effects of age and obesity on insulin binding to isolated adipocytes. *Endocrinology*, 96, 1486-97.

Orwin, D.F.G., Woods, J.L., Ranford, S.L. (1984). Cortical cell types and their distribution in wool fibres. *Australian journal of biological sciences*, 37, 237-55.

Orwin, D.F.G. (1989). Variations in wool follicle morphology. In *"The biology of wool and hair"*, 227-41. Rogers, G.E., Reis, P.J., Ward, K.A., Marshall, R.C., eds. Chapman and Hall, London and New York.

Orwin, D.F.G., Woods, J.L., Elliott, K.H. (1985). Composition of the cortex of sound and tender wools. *"Proceedings of the 6th international wool textile research conference, Pretoria"*, 2, 193-205.

Orwin, D.F.G., Woods, J.L., Elliott, K.H. (1980). Composition of the cortex of sound and tender wools. *Journal of the textile institute*, 71, 315-7.

Orwin, D.F.G., Woods, J.L., Gourdie, R.G. (1985). Cortical cell type and wool strength. *Proceedings of the 7th international wool textile research conference, Tokyo*, 1, 194-203.

Orwin, D.F.G, Gourdie, R.G., Woods, J.L., Geenty, K.G. (1987). Measuring staple

strength of New Zealand Romney and Corriedale wools. *New Zealand journal of experimental agriculture,* 15, 303-8.

Orwin, D.F.G., Woods, J.L. (1982). Number changes and developmental potential of wool follicle cells in the early stages of fibre differentiation. *Journal of ultrastructure research,* 80, 312-22.

Orwin, D.F.G., Geenty, K.G. (1986). Implications of objective measurement for fleece tenderness research. *Proceedings of the New Zealand society of animal production,* 46, 200-1.

Panaretto, B.A., Vickery, M.R. (1970). The rates of plasma cortisol entry and clearance in sheep before and during their exposure to a cold, wet environment. *Journal of endocrinology,* 47, 273-285.

Panaretto, B.A., Moore, G.P.M., Robertson, D.M., Bennett, J.W., Tunks, D.A. (1989). Biological wool harvesting. *Australian journal of biotechnology,* 3, (4), 258-63.

Panaretto, B.A., Leish, Z., Donnelly, J.B. (1982). Some effects of dexamethasone on nucleic acid metabolism in skin of Merino sheep. *Australian journal of biological sciences,* 35, 579-93.

Payne, J.M., Payne, S. (1987). *The metabolic profile test.* Oxford University Press.

Payne, E., Kendall, P., Hodgkinson, S. (1992). Binding of growth factors to nuclei and plasma membranes of ovine liver. *Proceedings of the Australian society of endocrinology,* 196.

Pell, M.M., Bergman, E.N. (1983). Cerebral metabolism of amino acids and glucose in fed and fasted sheep. *American journal of physiology,* 244, E282-9.

Phillips, L.S. (1986). Nutrition, somatomedins, and the brain. *Metabolism,* 35, (1), 78-87.

Philpott, M.P., Kealey, T. (1991). Metabolic studies on isolated hair follicles: hair follicles engage in aerobic glycolysis and do not demonstrate the glucose fatty acid cycle. *The journal of investigative dermatology,* 96, (6), 875-9.

Pilch, P.F., Czech, M.P. (1980). The subunit structure of the high affinity insulin receptor. *The journal of biological chemistry,* 255, (4), 1722-31.

Piper, L.R., Dolling, C.H.S. (1969). Efficiency of conversion of food to wool. V. Comparison of the apparent digestive ability of sheep selected for high clean wool weight with that of sheep from a random control group. *Australian journal of agricultural research,* 20, 579-87.

Piper, L.R., Dolling, C.H.S. (1966). Variation in the sulphur content of wool of Merino sheep associated with genetic differences in wool producing capacity. *Australian journal of biological sciences,* 19, 1179-82.

Pitt-rivers, R., Stanbury, J.B., Rapp, B. (1955). Conversion of thyroxine to 3-5-3'-triiodothyronine *in vivo. Journal of clinical endocrinology and metabolism,* 15, 616-20.

Podlecki, D.A., Frank, B.H., Kao, M., Horikoshi, H., Freidenberg, G., Marshall, S., Ciaraldi, T., Olefsky, J.M. (1983). Characterization of the receptor binding properties of monoiodoinsulin isomers and the identification of different insulin receptor specificities in hepatic and extrahepatic tissues. *Diabetes,* 32, 697-704.

Posner, B.I., Bergeron, J.J.M., Josefberg, Z., Khan, M.N., Khan, R.J., Patel, B. A., Sikstrom, R.A., Verma, A.K. (1981). Polypeptide hormones: intracellular receptors and internalization. *Recent progress in hormone research,* 37, 539-83.

Postic, C., Lecturque, A., Rencurel, F., Printz, R.L., Forest, C., Granner, D.K., Girard, J. (1993). The effects of hyperinsulinemia and hyperglycaemia on GLUT4 and hexokinase II mRNA and protein in rat skeletal muscle and adipose tissue. *Diabetes,* 42, 922-9.

Prior, R.L., Huntington, G.B., Reynolds, P.J. (1984). Role of insulin and glucose on metabolite uptake by the hind half of beef steers. *Journal of animal science,* 58, (6), 1446-53.

Purser, D.B. (1979). Effects of minerals upon wool growth. In *"Physiological and environmental limitations to wool growth"*, 243-55. Black, J.L., Reis, P.J. eds. University of New England publishing unit: Armidale, N.S.W.

Rechler, M.M., Podskalny, J.M. (1976). Insulin receptors in cultured human fibroblasts. *Diabetes,* 25, 250.

Reid, R.L. (1962). Studies on the carbohydrate metabolism of sheep XV. The adrenal response to the climatic stresses of cold, wind, and rain. *Australian journal of agricultural research,* 13, 296-306.

Reilly, P.E.B., Black, A.L. (1973). Early effects of cortisol on glucose and alanine metabolism in adrenalectomized sheep. *American journal of physiology,* 225, 689-95.

Reilly, P.E.B., Ford, E.J.H. (1974). The effect of betamethasone on glucose production and gluconeogenesis from amino acids in sheep. *Journal of endocrinology,* 60, 455-61.

Reis, P.J. (1979). Effects of amino acids on the growth and properties of wool. In *"Physiological and environmental limitations to wool growth"*, 223-42. Black, J.L., Reis, P.J. eds. University of New England publishing unit: Armidale, N.S.W.

Reis, P.J., Gillespie, J.M. (1985). Effects of phenylalanine and analogues of methionine and phenylalanine on the composition of wool and mouse hair. *Australian journal of biological sciences,* 38, 151-63.

Reis, P.J., Tunks, D.A., Downes, A.M. (1973). The influence of abomasal and intravenous supplements of sulphur-containing amino acids on wool growth rate. *Australian journal of biological sciences,* 26, 249-58.

Reis, P.J., Schinckel, P.G. (1964). The growth and composition of wool. II. The effect of casein, gelatin, and sulphur-containing amino acids given per abomasum.

Australian journal of biological sciences, 17, 532-47.

Reis, P.J. (1967). The growth and composition of wool. IV The differential response of growth and of sulphur content of wool to the level of sulphur-containing amino acids given per abomasum. *Australian journal of biological sciences,* 20, 809-25.

Reis, P.J. (1965). The growth and composition of wool III. Variations in the sulphur content of wool. *Australian journal of biological sciences,* 18, 671-87.

Reis, P.J., Tunks, D.A. (1978). Effects on wool growth of the infusion of mixtures of amino acids into the abomasum of sheep. *Journal of agricultural science,* 90, 173-83.

Reis, P.J. (1992). Variations in the strength of wool fibres - a review. *Australian journal of agricultural science,* 43, 1337-51.

Reis, P.J. (1975). Effects of intravenous infusion of mimosine on wool growth of Merino sheep. *Australian journal of biological sciences,* 28, 483-93.

Reis, P.J., Tunks, D.A., Downes, A.M. (1975). Fate of mimosine administered orally to sheep and its effectiveness as a defleecing agent. *Australian journal of biological sciences,* 28, 495-501.

Reis, P.J., Tunks, D.A., Williams, O.B., Williams, A.J. (1967). A relationship between sulphur content of wool and wool production by Merino sheep. *Australian journal of biological sciences,* 20, 153-63.

Reis, P.J., Chapman, R.E. (1974). Changes in wool growth and the skin of Merino sheep following administration of cyclophosphamide. *Australian journal of agricultural research,* 25, 931-43.

Reis, P.J., Schinckel, P.G. (1962). Some effects of sulphur-containing amino acids on the growth and composition of wool. *Australian journal of biological sciences,* 16, (1), 218-30.

Reis, P.J., Tunks, D.A., Rigby, R.D.G., Morton, T.C., Munro, S.G. (1983). Investigation of some amino acid analogues and metabolites as inhibitors of wool and hair growth. *Australian journal of biological sciences,* 36, 157-70.

Reitman, J.S., Vasquez, B., Klimes, I., Nagulesparan, M. (1984). Improvement of glucose homeostasis after exercise training in non-insulin-dependent diabetes. *Diabetes care,* 7, 434-41.

Ringberg Lund-Larsen, T., Bakke, H. (1975). Growth hormone and somatomedin activities in lines of pigs selected for rate of gain and thickness of backfat. *Acta agriculturae Scandinavica,* 25, 231-4.

Ringberg Lund-Larsen, T., Sundby, A., Kruse, V., Velle, W. (1977). Relation between growth rate, serum somatomedin and plasma testosterone in young bulls. *Journal of animal science,* 44, (2), 189-94.

Roberts, C.A., McCutcheon, S.N., Blair, H.T., Gluckman, P.D., Breier, B.H. (1991). Developmental patterns of plasma insulin-like growth factor-1

concentrations in sheep. *Domestic animal endocrinology,* 7 (4), 457-64.

Rogan, I.M. (1988-89). Genetic variation and covariation in wool characteristics related to processing performance and their economic significance. *Wool technology and sheep breeding,* 126-35.

Rogers, G.E., Kuczek, E.S., MacKinnon, P.J., Presland, R.B., Fietz, M.J. (1989). Special biochemical features of the hair follicle. In *"The biology of wool and hair"*, 69-85. Rogers, G.E., Reis, P.J., Ward, K.A., Marshall, R.C. eds, Chapman and Hall, London and New York.

Rogers, G.R., Orwin, D.F.G., Fraser, M.C., Bray, A.R., Smith, M.C., Baird, D.B., Clarke, J.N., Bickerstaffe, R. (1990). Selective breeding for high and low strength Romney wools. *Proceedings of the 8th international wool textile conference,* 2, 180-8.

Ross, D.A. (1982). Staple length, staple strength, fibre length and processing. *Wool,* 7, (4), 12-5.

Ross, D.A. (1965). Wool growth of the New Zealand Romney. *New Zealand journal of agricultural research,* 8, 585-601.

Ross, D.A. (1984). Physical factors affecting the staple-strength measurement. In *"Staple strength. Report of a seminar on staple strength held at Lincoln College, August, 1984. Lincoln College wool bulletin"*, 29-33.

Roth, R.A., Maddux, B., Wong, K.Y., Styne, D.M., Van Vliet, G., Humbel, R.E., Goldfine, I.D. (1983). Interactions of a monoclonal antibody to the insulin receptor with receptors for insulin-like growth factors. *Endocrinology,* 112, (5), 1865-7.

Rothman, S. (1954). In *"Physiology and biochemistry of the skin"*, p32. The University of Chicago Press.

Rowe, J.B., Brown, G., Ralph, I.G., Ferguson, J., Wallace, J.F. (1989). Supplementary feeding of young Merino sheep, grazing wheat stubble, with different amount of lupin, oat or barley grain. *Australian journal of experimental agriculture,* 29, 29-35.

Ryder, M.L. (1955). Studies on the nutrition of wool follicles in sheep: The anatomy of the general blood supply to the skin. *The journal of agricultural science,* 45, 311-26.

Sadler, W.A., Brownley, B.E.W. (1975). Triiodothyronine radioimmunoassay in the assessment of thyroid function. *New Zealand medical journal,* 81, 328-34.

Salem, M.H., El-Sherbiny, A.A., Khalil, M.H., Yousef, M.K. (1991). Diurnal and seasonal rhythm in plasma cortisol, triiodothyronine and thyronine as affected by the wool coat in Barki sheep. *Indian journal of animal sciences,* 61, (9), 946-51.

Saltiel, A.R. (1990). Second messengers of insulin action. *Diabetes care,* 13, (3), 244-56.

Sarkar, H.K., Thorens, B., Lodish, H.F., Kaback, H.R. (1988). Expression of the

human erythrocyte glucose transporter in *Escherichia coli. Proceedings of the national academy of sciences, USA,* 85, 5463-7.

Sasaki, Y., Takahashi, H., Aso, A., Ohneda, A., Weekes, T.E.C. (1982). Effect of cold exposure on insulin and glucagon secretion in sheep. *Endocrinology,* 111, 2070-6.

Sasaki, Y., Weekes, T.E.C. (1986). Metabolic responses to cold. In *" Control of digestion and metabolism in ruminants"*, 326-43. Milligan, L.P., Grovum, W.L., Dobson, A. eds. Prentice Hall: Englewood Cliffs NJ 07632.

Sasaki, Y., Weekes, T.E.C., Bruce, J.B. (1977). Effects of glucose and butyrate on insulin release from perifused fragments of sheep pancreas. *Journal of endocrinology,* 72, 415-6.

Sasaoka, T., Takata, Y., Kusari, J., Anderson, C.M., Langlois, W.J., Olefsky, J.M. (1993). Transmembrane signaling by an insulin receptor lacking a cytoplasmic beta-subunit domain. *Proceedings of the national academy of sciences, USA,* 90, (10), 4379-83.

Saul, G.R., Channon, H.A. (1990). Feed intake, liveweight gain and wool growth of Merino wethers fed grass or legume hays. *Proceedings of the Australian society of animal production,* 18, 545.

Scatchard, G. (1949). The attraction of proteins for small molecules and ions. *Annals of the New York Academy of Sciences,* 51, 660-672.

Schinckel, P.G. (1961). Mitotic activity in wool follicle bulbs. *Australian journal of biological sciences,* 14, 659-76.

Schober, D.A., Simmen, F.A., Hadsell, D.L., Baumrucker, C.R. (1990). Perinatal expression of type I IGF receptors in porcine small intestine. *Endocrinology,* 126, 1125.

Schwander, J.C., Hauri, C., Zapf, J., Froesch, E.R. (1983). Synthesis and secretion of insulin like growth factor and its binding protein by the perfused rat liver: dependence on growth hormone status. *Endocrinology,* 113, 297.

Scobie, D.R., Woods, J.L. (1992). A review implicating a two compartment model for the processes of cell division and differentiation in the wool follicle. *Proceedings of the New Zealand society of animal production,* 52, 265-71.

Scott, I.C., Heath, D., Hawker, H. (1992). Melatonin secretion in Romney ewes differing in wool growth and reproduction is not aligned to photoperiod during spring and summer. *Proceedings of the New Zealand society of animal production,* 52, 149-52.

Shows, T.B., Eddy, R.L, Byers, M.G., Fukushima, Y., Dehaven, C.R., Murray, J.C., Bell, G.I. (1987). Polymorphic human glucose transporter gene (GLUT) is on chromosome 1p 31.3-p35. *Diabetes,* 36, 546-9.

Shutt, D.A, Fell, L.R., Connell, R., Bell, A.K., Wallace, C.A., Smith, A.I. (1987). Stress-induced changes in plasma concentrations of immunoreactive B-endorphin and cortisol in response to routine surgical procedures in

lambs. *Australian journal of biological sciences,* 40, 97-103.

Siess, E., Teinzer, A., Struck, E., Wieland, O. (1965). Bilding eines insulinartigen Wirkstoffes durch die isolierte Rattenleber. *Diabetologia,* 1, 21-7.

Sivitz, W.I., DeSautel, S.L., Kayano, T., Bell, G.I., Pessin, J.E. (1989). Regulation of glucose transporter messenger RNA in insulin-deficient states. *Nature,* 340, 72-4.

Skolnik, E.Y., Batzer, N., Li, C.-H., Lowenstein, E., Mohammadi, M., Margolis, B., Schlesssinger, J. (1993). The function of GRB2 in linking the insulin receptor to *Ras* signaling pathways. *Science,* 260, 1953-5.

Slen, S.B., Connell, R. (1958). Wool growth in sheep as affected by the administration of certain sex hormones. *Canadian journal of animal science,* 38, 38-47.

Slot, J.W., Geuze, H.J., Gigengack, S., James, D.E., Lienhard, G.E. (1991). Translocation of the glucose transporter GLUT4 in cardiac myocytes of the rat. *Proceedings of the national academy of sciences, USA,* 88, (17), 7815-9.8

Stadmauer, L., Rosen, O.M. (1986). Increasing the cAMP content of IM-9 cells alters the phosphorylation state and protein kinase activity of the insulin receptor. *The journal of biological chemistry,* 261, 3402.

Steele, N.C., Elsasser, T.H. (1989). Regulation of somatomedin production release, and mechanisms of action. In *"Animal growth regulation",* 295-311. Campion, D.R., Hausman, G.J., Martin, R.J., eds. Plenum press, New York and London.

Steinert, P.M., Torchia, D.R., Mack, J.W. (1989). Structural features of keratin intermediate filaments. In *"The biology of wool and hair",* 157-68. Rogers, G.E.,Reis, P.J. Ward K.A., Marshall, R.C. eds. Chapman and Hall, London New York.

Stephenson, R.G.A., Suter, G.R., Howitt, C.J. (1991). Wool growth responses to DL-methionine administration and factors affecting the value of supplementation. *Australian journal of experimental agriculture,* 31, (4), 471-7.

Sterling, K., Lazarus, J.H. (1977). The thyroid and its control. *Annual review of physiology,* 39, 349-71.

Story, L.F., Ross, D.A. (1959). Effect of shearing time on wool. V. Effect on processing. *New Zealand journal of agricultural research,* 2, 1096-1103.

Story, L.F., Ross, D.A. (1960). Effect of shearing time on wool. IV. The rate of growth of wool and its relation to time of shearing. *New Zealand journal of agricultural research,* 3, 113-24.

Strowitzki, T., von Eye, H.C., Kellerer, M., Haring, H.U. (1993). Tyrosine kinase activity of insulin-like growth factor I and insulin receptors in human endometrium during the menstrual cycle: Cyclic variation of insulin receptor expression. *Fertility and sterility,* 59, (2), 315-22.

Sugden, P.H., Fuller, S.J. (1991). Regulation of protein turnover in skeletal and

cardiac muscle. *Biochemical journal,* 273, 21-37.

Sumner, R.M.W., Hawker, H. (1986). Implications of objective measurement for sheep management. *Proceedings of the New Zealand society of animal production,* 46, 198-200.

Sumner, R.M.W. (1983). Effects of feeding and season on fleece characteristics of Cheviot, Drysdale and Romney hogget wool. *Proceedings of the New Zealand society of animal production,* 43, 79-82.

Sun, Y.X., Matthew, T.C., Blair, H.T., Harris, P.M., McCutcheon, S.N. (1992). Seasonal variation in plasma metabolite and hormone concentrations, amino acid utilization and responses to thyrotropin-releasing hormone in fleeceweight-selected and control Romney rams. *Australian journal of agricultural research,* 43, 1845-58.

Sun, Y.X., Michel, A., Wickham, G.A., McCutcheon, S.N. (1992). Wool follicle development, wool growth and body growth in lambs treated from birth with recombinantly derived bovine somatotropin. *Animal production,* 55, 73-8.

Suttie, J.M., White, R.G., Manley, T.R., Breier, B.H., Gluckman, P.D., Fennessy, P.F., Woodford, K. (1993). Insulin-like growth factor I and growth seasonality in reindeer (*Rangifer tarandus*) - comparisons with temperate and tropical cervids. *Rangifer,* 13, 91-7.

Tabor, C.W., Tabor, H. (1976). 1,4-diaminobutane (putrescine), spermidine, and spermine. *Annual review of biochemistry,* 45, 285-306.

Taylor, S.I., Najjar, S., Cama, A., Accili, D. (1991). Structure and function of the insulin receptor. In *"Insulin-like growth factors: Molecular and cellular aspects"*, 221-44. LeRoith, D., ed, CRC Press, Boca Raton Ann Arbor Boston London.

Taylor, S.I., Kadowaki, T., Accili, D., Cama, A., Kadowaki, H., McKeon, C., Moncada, V., Marcus-Samuels, B., Bevins, C., Ojamaa, K., Frapier, C., Beitz, L., Perrotti, N., Rees-Jones, R., Margolis, R., Imano, E., Najjar, S., Courtney, F., Arakaki, R., Gorden, P. Roth, J. (1990). Mutations in the insulin receptor gene in genetic forms of insulin resistance. *Recent progress in hormone research,* 46, 185-217.

Tenenhouse, H.S., Gold, R.J.M., Kachra, Z. (1974). Biochemical marker in dominantly inherited ectodermal malformation. *Nature,* 251, 431-2.

Thomson, B.C., Dellow, D.W., Barry, T.N. (1989). The effect of selection for fleece weight upon urea metabolism and digestive function in Romney sheep. *Australian journal of agricultural research,* 40, 1065-74.

Thorens, B., Charron, M.J., Lodish, H.F. (1990). Molecular physiology of glucose transporters. *Diabetes care,* 13, (3), 209-17.

Thorens, B. (1993). Facilitated glucose transporters in epithelial cells. *Annual reviews in physiology,* 55, 591-68.

Thurley, D.C., McNatty, K.P. (1973). Factors affecting peripheral cortisol levels in unrestricted ewes. *Acta endocrinologica,* 74, 331-7.

Tordjman, K.M., Leingang, K.A., James, D.E., Mueckler, M.M. (1989). Differential regulation of two distinct glucose transporter species expressed in 3T3-L1 adipocytes: Effect of chronic insulin and tolbutamide treatment. *Proceedings of the national academy of sciences, USA,* 86, 7761-5.

Ullrich, A., Gray, A., Tam, Q.W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J., Fujita-Yamaguchi, Y. (1986). Insulin-like growth factor I receptor primary structure: Comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO journal,* 5, 2503-12.

Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P.H., Grunfeld, C., Rosen, O.M., Ramachandran, J. (1985). Human insulin receptor and its relationship to the tyrosine kinase. *Nature,* 313, 757-61.

Van Luijk, C. (1984). Theory of staple strength measurement. In *"Report of a seminar on staple strength. Lincoln College, New Zealand, 1984"*, 17-23. D.A. Ross, Story, L.F. eds. Lincoln College wool bulletin.

Van Wyk, J.J., Graves, D.C., Casella, S.J., Jacobs, S. (1985). Evidence from monoclonal antibody studies that insulin stimulates deoxyribonucleic acid synthesis through the Type I somatomedin receptor. *Journal of clinical endocrinology and metabolism,* 61, (4), 639-49.

Verrando, P., Ortonne, J.P. (1985). Insulin binding properties of normal and transformed human epidermal cultured keratinocytes. *The journal of investigative dermatology,* 85, (4), 328-32.

Walker, P.S., Ramlal, T., Sarabia, V., Koivisto, U.M., Bilan, P.J., Pessin, J.E., Klip, A. (1990). Glucose transport activity in L6 muscle cells is regulated by the coordinate control of subcellular glucose transporter distribution, biosynthesis, and mRNA transcription. *The journal of biological chemistry,* 265, (3), 1516-23.

Wallace, A.L.C. (1979). The effect of hormones on wool growth. In *"Physiological and environmental limitations to wool growth"*, 257-68. Black, J.L., Reis, P.J. eds. University of New England publishing unit: Armidale, N.S.W.

Ward, K.A., Harris, R.L.N. (1976). Inhibition of wool follicle DNA synthesis by mimosine and related 4(1H)-pyridones. *Australian journal of biological sciences,* 29, 189-96.

Wastney, M., Arcus, A.C., Bickerstaffe, R., Wolff, J.E. (1982). Glucose tolerance in ewes and susceptibility to pregnancy toxaemia. *Australian journal of biological sciences,* 35, 381-92.

Weekes, T.E.C. (1991). Hormonal control of glucose metabolism. In *"Physiological aspects of digestion and metabolism in ruminants: Proceedings of the seventh international symposium on ruminant physiology"*, 183-200. Tsuda, T., Sasaki,

Y., Kawashima, R. eds. Academic Press, Inc.

Werner, H., Shen-Orr, Z., Stannard, B., Burguera, B., Roberts, C.T., Jr, LeRoith, D. (1990). Experimental diabetes increases insulin-like growth factor I and II receptor concentrations and gene expression in the kidney. *Diabetes,* 39, 1490.

White, C.L., Martin, G.B., Hynd, P.I., Chapman, R.E. (1994). The effect of zinc deficiency on wool growth and skin and wool follicle histology of male Merino lambs. *British Journal of Nutrition,* 71, 425-435.

Wieghart, M., Slepetis, R., Elliot, J.M., Smith, D.F. (1986). Glucose absorption and hepatic gluconeogenesis in dairy cows fed diets varying in forage content. *Journal of nutrition,* 116, 839-50.

Wiggins, L.K. (1986). Benefits and incentives available from objective measurement. *Proceedings of the New Zealand society of animal production,* 46, 186-9.

Williams, A.J., Robards, G.E., Saville, D.G. (1972). Metabolism of cystine by Merino sheep genetically different in wool production II. The responses in wool growth to abomasal infusions of L-cysteine or DL-methionine. *Australian journal of biological sciences,* 25, 1269-76.

Williams, A.J., Winston, R.J. (1987). A study of the characteristics of wool follicle and fibre in Merino sheep genetically different in wool production. *Australian journal of agricultural research,* 38, 743-55.

Williams, A.J., Butt, J. (1989). Wool growth of pregnant Merino ewes fed to maintain maternal liveweight. *Australian journal of experimental agriculture,* 29, (4), 503-7.

Willis, J.A. (1990). Insulin status and carcass composition of lambs: Effects of weaning age and treatment with exogenous insulin. *Ph D thesis, Lincoln University, Canterbury, New Zealand.*

Wilson, P.A., Short, B.F. (1979). Cell proliferation and cortical cell production in relation to wool growth. *Australian journal of biological sciences,* 32, 317-27.

Woods, J.L., Orwin, D.F.G. (1987). Wool proteins of New Zealand Romney sheep. *Australian journal of biological sciences,* 40, 1-14.

Wynn, P.C., Wallace, A.L.C., Kirby, A.C., Annison, E.F. (1988). Effects of growth hormone administration on wool growth in Merino sheep. *Australian journal of biological sciences,* 41, 177-87.

Wynn, P.C., Searle, T.W., Graham, N.M., Baker, P., Fleck, E., Ward, W. (1990). The relationship between live weight, body composition and endocrine function in Border Leicester X Merino wethers. *Proceedings of the Australian society of animal production,* 18, 568.

Yamaguchi, Y., Flier, J.S., Benecke, H., Ransil, B.J., Moller, D.E. (1993). Ligand-binding properties of the two isoforms of the human insulin receptor. *Endocrinology,* 132, (3), 1132-8.

Yamamoto-Honda, R., Kadowaki, T., Momomura, K., Tobe, K., Tamori, Y., Shibasaki, Y., Mori, Y., Kaburagi, Y., Koshio, O., Akanuma, Y. et al. (1993). Normal insulin receptor substrate-1 phosphorylation in autophosphorylation-defective truncated insulin receptor. Evidence that phosphorylation of substrates might be sufficient for certain biological effects evoked by insulin. *Journal of biological chemistry,* 268, (22), 16859-65.

Yeoh, S.I., Baxter, R.C. (1988). Metabolic regulation of the growth hormone independent insulin-like growth factor binding protein in human plasma. *Acta endocrinologica,* 119, 465-73.

Yki-Jarvin, H., Vuorinen-Markkola, H., Koranyi, L., Bourey, R., Tordjman, K., Mueckler, M., Permutt, A.M., Koivisto, A. (1992). Defect in insulin gene expression of the muscle/adipose tissue glucose transporter gene in skeletal muscle of Type I diabetic patients. *Journal of clinical endocrinology and metabolism,* 75, (3), 795-9.

Yonezawa, K., Ando, H., Kaburagi, Y., Yamamoto-Honda, R., Kitamura, T., Hara, K., Nakafuku, M., Okabayashi, Y., Kadowaki, T., Kaziro, Y., Kasuga, M. (1994). Signal transduction pathways from insulin receptors to *Ras.* Analysis by mutant insulin receptors. *Journal of biological chemistry,* 269, (6), 4634-40.

Young, R.I., Field, B.W. (1988). Mechanical defleecing of biologically weakened wool. *Agricultural engineering Australia,* 17, (2), 26-31.

Zainur, A.S., Tassell, R., Kellaway, R.C., Dodemaide, W.R. (1989). Recombinant growth hormone in growing lambs: Effects on growth, feed utilization, body and carcass characteristics and on wool growth. *Australian journal of agricultural research,* 40, 195-206.

Zhao, F.Q., Glimm, D.R., Kennelly, J.J. (1993). Distribution of mammalian facilitative glucose transporter messenger RNA in bovine tissues. *International journal of biochemistry,* 25, (12), 1897-1903.

Zierler, K. (1989). Misuse of nonlinear Scatchard plots. *TIBS,* 14, 314-7.

Zierler, Z. (1985). Actions of insulin on electrical potential differences across cell membranes. *In "Molecular basis of insulin action",* 119-134. Czech, M.P., ed. Plenum Press: New York, London.

Chemicals

Agar.	Davis agar, DAVIS GELATINE (N.Z.) LTD.
Agarose.	Product 50002,Seakem, LE, FMC BIOPRODUCTS.
Ammonium sulphate.	Product 10033, AnalaR, BDH CHEMICALS LTD.
Ampicillin.	Product 835242, BOEHRINGER MANNHEIM NZ LTD.
Amyl alcohol.	Product 2525, Pronalys, May and Baker, RHONE POULENC NEW ZEALAND LTD.
Aprotinin.	Product A6279, SIGMA CHEMICAL COMPANY.
Bacitracin.	Product B0125, SIGMA CHEMICAL COMPANY.
Boric acid (crystallized).	Product 20 185.297. Normapur AR. RHONE POULENC NEW ZEALAND LTD.
Bovine insulin, crystalline.	GIBCO BRL, LIFE TECHNOLOGIES.
Bovine serum albumin. (Fraction V)	Product A8022, SIGMA CHEMICAL COMPANY.
Bovine insulin antiserum.	Product I6136, SIGMA CHEMICAL COMPANY.
Bromophenol Blue.	Product 20017, BDH CHEMICALS LTD.
Calcium chloride.	Product 26224, BDH CHEMICALS LTD.
Casein hydrolysate (Peptone No 140).	Product 152-00014M, GIBCO BRL, LIFE TECHNOLOGIES.
Charcoal (activated - not acid washed).	Product 33032, BDH CHEMICAL COMPANY.
Chloroform.	Product 27710, BDH CHEMICALS LTD.

DEPC. (diethyl pyrocarbonate).	Product D5758, SIGMA CHEMICAL COMPANY.
Dextran sulphate (sodium salt).	Product 17-0340-01, PHARMACIA DIAGNOSTICS LTD.
Dextran. (MW 60000-90000).	Product D4751, SIGMA CHEMICAL COMPANY.
Di-potassium hydrogen orthophosphate.	Product 29619, BDH CHEMICALS LTD.
EDTA (ethylenediaminetetraacetic acid disodium salt).	Product 808270, BOEHRINGER MANNHEIM NZ LTD.
Deoxyribonucleic acid (from salmon sperm). Sodium salt Type III.	Product D1626, SIGMA CHEMICAL COMPANY.
Ethanol, absolute.	Product EL975, Pronalys, RHONE POULENC NEW ZEALAND LTD.
Ethidium bromide.	Product E8751, SIGMA CHEMICAL COMPANY.
Ficoll.	Product F4375, SIGMA CHEMICAL COMPANY.
Formaldehyde solution 40% w/v.	Pronalys, Product L909A, May and Baker, RHONE POULENC NEW ZEALAND LTD.
Formamide.	Product F-7503, SIGMA CHEMICAL COMPANY.
Glacial acetic acid.	Product AL723, RP Pronalys AR, RHONE POULENC NEW ZEALAND LTD.
Glucose.	Product 10117, AnalaR, BDH CHEMICALS LTD.
Glycerol.	Product GL885, Pronalys, RHONE POULENC NEW ZEALAND LTD.
Guanidine (aminomethanamidine) Thiocyanate salt.	Product G6639, SIGMA CHEMICAL COMPANY.
Human serum albumin.	Product ORHA21, BEHRING DIAGNOSTICS.

Lithium chloride.	Product 29067, BDH CHEMICALS LTD.
Magnesium sulphate.	Product 10151, AnalaR, BDH CHEMICALS LTD.
Magnesium chloride.	Product 296, Univar, AJAX CHEMICALS LTD.
2-Mercaptoethanol.	Product M3148, SIGMA CHEMICAL COMPANY.
MOPS (4-morpholinepropanesulfonic acid).	Product 1124676, BOEHRINGER MANNHEIM NZ LTD.
Ovine insulin, crystalline.	Product I9254, SIGMA CHEMICAL COMPANY.
Phenyl methyl sulphonyl fluoride (PMSF)	Product P7626, SIGMA CHEMICAL COMPANY.
Polyethylene glycol. (approx. MW 8000).	Product P2139, SIGMA CHEMICAL COMPANY.
Potassium acetate.	Product 10350, AnalaR, BDH CHEMICALS LTD.
Potassium dihydrogen phosphate.	Product 26 926.298, Normapur, RHONE POULENC NEW ZEALAND LTD.
Potassium chloride.	Product 26 764.298, Normapur, RHONE POULENC NEW ZEALAND LTD.
Pronase. (From *Streptomyces griseus*)	Product 165921, BOEHRINGER MANNHEIM NZ LTD.
Propan-2-ol.	Product 10224, AnalaR, BDH CHEMICALS LTD.
RNAse (DNAse free).	Product 1119 915, BOEHRINGER MANNHEIM NZ LTD.
Sarcosyl sodium salicylate	Product 28 068.296, Rectapur, RHONE POULENC NEW ZEALAND LTD.
SDS	Product 20760 ,SERVA FEINBIOCHEMICA

(dodecylsulfate-Na-salt crystalline).	GmbH & CO.
Sephadex G-50.	Product 17-0043-01, PHARMACIA
(tri-sodium citrate-2-hydrate).	DIAGNOSTICS LTD.
Sodium acetate (anhydrous).	Product S2889, SIGMA CHEMICAL COMPANY.
Sodium dihydrogen phosphate.	Product 10245, AnalaR, BDH CHEMICALS LTD.
Sodium chloride.	Product 895-18301Q, GIBCO BRL, LIFE TECHNOLOGIES.
Sodium citrate.	Product 25116, SCIENTIFIC SUPPLIES LTD.
Sodium hydroxide (pellets).	Product 10252, AnalaR, BDH CHEMICALS LTD.
Sucrose.	Product 10274, AnalaR, BDH CHEMICALS LTD.
TRIS	Product 708 976, BOEHRINGER
(2-amino-2-(hydroxymethyl)-1,3-propanediol).	MANNHEIM NZ LTD.
TRIZMA hydrochloride.	Product T7149, SIGMA CHEMICAL COMPANY.
Urea.	Product U5378, SIGMA CHEMICAL COMPANY.
Xylene Cyanol FF.	Product 28 986.130, May and Baker, RHONE POULENC NEW ZEALAND LTD.
Yeast extract.	Product 152-00047M, GIBCO BRL, LIFE TECHNOLOGIES.

Distributors

AJAX CHEMICALS LTD, PO Box 14-147, Auckland 6, New Zealand.

AMERSHAM AUSTRALIA PTY. LTD, New Zealand Branch, PO Box 6261, Wellesley St, Auckland, New Zealand.

BDH CHEMICALS LTD, BDH Laboratory Supplies, Poole BH15 ITD, England.
Distributed by: BIOLAB SCIENTIFIC, PO Box 1813, Christchurch, New Zealand.

BEHRING DIAGNOSTICS, Behringwerke AG Diagnostica, Postfach 1140, D-3550 Marburg 1.Distributed by: HOECHST NEW ZEALAND LTD, PO Box 67, Panmure, Auckland.

BIO-RAD LABORATORIES PTY. LTD, P.O. Box 100-051, Auckland, New Zealand.

BOEHRINGER MANNHEIM NZ LTD, PO box 62-089, Auckland, New Zealand.

DAVIS GELATINE (N.Z.) LTD,
Connal St, Woolston, Christchurch, New Zealand.

FMC BIOPRODUCTS, Rockland ME04841, USA.
Distributed by: BAXTER DIAGNOSTICS, PO Box 14-062, Panmure Auckland.

GIBCO BRL - LIFE TECHNOLOGIES, PO Box 12-502, Penrose, Auckland 6, New Zealand.

NEW ENGLAND BIOLABS, INC, Beverley MA01915-5599, USA.
Distributed by: BIOLAB SCIENTIFIC, PO Box 1813, Christchurch, New Zealand.

PHARMACIA DIAGNOSTICS LTD, Uppsala, Sweden.
Distributed by: NZ MEDICAL & SCIENTIFIC LTD, PO Box 24-138, Royal Oak, New Zealand.

RHONE POULENC NEW ZEALAND LTD, PO Box 35-060, Naenae, Lower Hutt, New Zealand. Distributed by: NDA LABWARE, PO Box 9033, Christchurch, New Zealand.

SERVA FEINBIOCHEMICA GMBH & CO, PO Box 105260, D-6900, Heidelberg 1.
Distributed by SCIENTIFIC SUPPLIES LTD, PO Box 14-454, Panmure, Auckland, New Zealand.

SIGMA CHEMICAL COMPANY, PO Box 14508, St Louis, MO 63178 U.S.A.
Distributed by: BIOLAB SCIENTIFIC, PO Box 1813, Christchurch, New Zealand.

TERUMO CORPORATION, Tokyo, Japan. Distributed by: BIOLAB SCIENTIFIC, PO Box 1813, Christchurch, New Zealand.

Radio-immuno-assay (RIA) for ovine insulin

1. Reagents

Phosphate buffer

4.45 g NaH_2PO_4
17.25 g Na_2HPO_4

Make up to 1 litre and adjust pH to 3.4.

BSA buffers

Made by dissolving the appropriate amount of bovine serum albumin in phosphate buffer.

Blank plasma

Insulin was removed from blood plasma by the addition of 1 % charcoal and mixing on ice for 1 hour. The charcoal was consequently removed by a 2000 g 30 minute centrifugation step and the 'blank plasma' decanted off. Plasma was stored at -20 °C until required.

2. Iodination

10 µl ^{125}I (100 µCi/µl in 0.1 M NaOH) was added to 5 µl bovine insulin (1 mg/ml in 0.01 M HCl) and 10 ul 0.3 M sodium phosphate buffer. The reaction was started by the addition of 17 ul of chloramine T (40 µg/ml prepared in sodium phosphate buffer immediately prior to use and stored shielded from light). The reaction was stopped after 90 seconds by the addition of 100 µl 2.5 % BSA buffer.

3. Estimation of incorporation of I125 into insulin

2 ul of the reaction mixture was diluted with 1 ml 0.3% BSA buffer. Triplicate 10 µl aliquots of the diluted reaction mixture were counted using an LKB compugamma. One ml 0.3 % HSA buffer and 1 ml TCA (10% prepared in distilled water) were added to each tube following counting and prior to centrifugation for 1 minute at 2300 g. The precipitate was counted and the counts were expressed as the percentage of the counts present before TCA precipitation. This value represented the incorporation of ^{125}I into the insulin. Purification of insulin was performed if at least 40 % of the radioactivity was precipitated by TCA.

4. Purification of iodinated insulin

Iodinated insulin was purified using gel filtration chromatography. 2.09 g Sephadex-G25 was swollen with 2.09 g Sephadex-G50 overnight in excess 0.1 % BSA buffer. The slurry was poured into a column of 40 cm in length and 1 cm in diameter. A solution containing potassium dichromate and blue dextran was added to the reaction mixture and after application to the column fractions containing iodinated insulin were eluted between the dextran and dichromate peaks. The fraction with the highest counts was retained for radioimmunoassay procedures.

5. Radioimmunoasssay

1. Bovine insulin antiserum (Sigma) was diluted in 0.3 % HSA according to the manufacturer's instructions.

2. Standards were prepared ranging from 0.33 µU to 200 µU using ovine insulin (Sigma) dissolved in 0.3% HSA phosphate buffer.

3. Tubes were prepared containing 100 µl of standard, control sample, blank plasma, 0.3% BSA phosphate buffer or test sample.

4. Tubes were vortexed and incubated for 5 hours at 4 °C.

5. 100 µl of tracer was added so that 10 000 cpm were present per tube.

6. Tubes were vortexed and incubated for 18-20 hours at 4 °C.

7. 100 µl of blank plasma was added to tubes containing buffer or standard.

8. A 2.5 % charcoal in 0.3% BSA phosphate buffer solution was prepared by stirring the charcoal into the buffer for 30 minutes while on ice. 500 µl of this charcoal solution was added to each tube except the totals.

9. Tubes were vortexed and centrifuged at 3000 rpm at 4 °C for 15 minutes.

10. Samples were aspirated and the pellet counted by a LKB Compu Gamma counter for 3 minutes.

Genstat program for the calculation of T-half

```
'REFER/NID=60,NUNN=30' GLUCOSE_TOLERANCE_TEST
'UNITS'  $ 86
'heading' outfile = ''LGTT.out''
'output/file=outfile'  2  $ 132
'READ/P'  TAG,T(1...5),GLC(1...5)
'SCALAR'  PT(1)=0 : PT(2)=20 : PT(3)=40 : PT(4)=60 : PT(5)=90
'CALC'    T(1...5)   = INTPT(T(1...5))+(T(1...5)-INTPT(T(1...5)))*100/60
  :       T(2...5)   = T(2...5) - T(1)
  :       GLC(2...5) = LOG(GLC(2...5))
  :       REG2090    = VREG(T(2...5);GLC(2...5))
  :       Thalf      = - LOG(2)/REG2090
'PRINT/P' TAG,GLC(1),Thalf
          $ 3,7.1,9.1
'RUN'
/* DATA e.g.

Tag t=0 t=20 t=40 t=60 t=90 glucose0 glucose20 glucose40 glucose60 glucose90

*/
'EOD'
'CLOSE'
'STOP'
```

SAS program for the calculation of insulin tolerance

```
** Written by David Baird, AgResearch, PO Box 60, Lincoln, New Zealand, 1991**
** In SAS/PC 6.04 **


LIBNAME NEW '.';

OPTIONS LINESIZE = 80 PAGESIZE = 63 NOCENTER ;

DATA NEW.ITT ;
    TITLE 'APRIL 1990 INSULIN TOLERANCE TEST DATA' ;
    INFILE 'ITT.TXT' ;
    LABEL TAG       = 'Tag'
          REALTIME = 'Bleeding Time'
          THEOTIME = 'Theoretical Bleeding Time'
          GLUCOSEA = 'Glucose from autoanalyzer'
          GLUCOSEB = 'Glucose from Beckman' ;
    FORMAT THEOTIME TIME5. REALTIME TIME8. ;
    INPUT ANIMAL 1-3 TAG 6-8 +2 THEOTIME TIME8. +2 REALTIME TIME8.
          GLUCOSEA 31-35 GLUCOSEB 41-42 ;
RUN ;

PROC PLOT DATA = NEW.ITT NOMISS ;
    PLOT GLUCOSEA*REALTIME='*' GLUCOSEB*REALTIME='+' / OVERLAY ;
    BY TAG ;
RUN ;

PROC REG DATA = NEW.ITT
        OUTEST = NEW.ITTEST (KEEP = ANIMAL TAG INTERCEP REALTIME
_DEPVAR_ ) ;
    MODEL GLUCOSEA GLUCOSEB = REALTIME ;
    OUTPUT OUT = NEW.ITTOUT P = PREDGLUC R = DEVIAT_N ;
    BY ANIMAL TAG ;
RUN ;

DATA NEW.ITTEST ;
    TITLE 'APRIL 1990 INSULIN TOLERANCE TEST DATA REGRESSION
ESTIMATES' ;
    SET NEW.ITTEST ;
    RENAME _DEPVAR_ = GFORM ;
    LABEL INTERCEP = 'Intercept'
          GFORM    = 'Glucose measurement' ;
    FORMAT REALTIME 8.5 ;
```

```
    REALTIME = REALTIME*60 ;
RUN ;

PROC SORT DATA = NEW.ITTEST ; BY TAG GFORM ; RUN ;

PROC PRINT DATA = NEW.ITTOUT NOOBS ;
    ID TAG ;
RUN ;

PROC PRINT DATA = NEW.ITTEST NOOBS ;
    ID TAG ;
    VAR ANIMAL GFORM INTERCEP REALTIME ;
RUN ;
```

SAS program for the transformation of insulin binding data

```
/* Program to Pre-process data for input into GAUSS SCATCHXY program      **
** Written by David Baird, AgResearch PO Box 60, Lincoln, New Zealand, 1992 **
** in SAS/PC 6.04                                                        */

options linesize=78 ;

%window device irow=11 icolumn=22 rows=9 columns=45 color=blue
 #1  @3 'Choose output device: ' c=yellow
 #3  @8 '1. Screen. ' c=yellow
 #4  @8 '2. Laser printer.' c=yellow
 #6  @6 'Output device (1/2) ? ' c=yellow dno 1 c= white ;

%window File irow=1 icolumn=22 rows=7 columns=45 color=blue
 #1  @3 'Enter the name of the file to process' c=yellow
 #2  @5 '(leave of the file extension .dat)' c=yellow
 #4  @5 'Filename = ? ' c=yellow fname 8 c=white ;

%macro cpm(file) ;
%local pdevice screen ;
%let screen = &sysdevic. ;
%let dno =  ;

data cpm ;
    infile "&file..dat" ;
    length tag $8 ;
    retain tag " " hotcpm volume nrep nsample coldconc sample rep . ;
    input  tag $ hotcpm volume nrep nsample ;
    put    tag= hotcpm= volume= nrep= nsample= ;
    do sample = 1 to nsample ;
      input coldconc @@ ;
      do rep = 1 to nrep ;
        input coldcpm @@ ;
        if coldcpm ^= . then output ;
      end ;
    end ;
    input ; /* Skip blank line */
run ;

proc sort data = cpm ; by tag descending coldconc rep ; run ;

data cpm ;
    retain maxcold ncoldcpm scoldcpm nsbind . ;
```

```
    drop ncoldcpm scoldcpm ;
    set cpm ; by tag descending coldconc ;
    if first.tag then do ;
      maxcold = coldconc ;
      ncoldcpm = 0 ;
      scoldcpm = 0 ;
      nsbind = . ;
    end ;
    if coldconc = maxcold then do ;
      if coldcpm ^= . then do ;
        scoldcpm = scoldcpm + coldcpm ;
        ncoldcpm = ncoldcpm + 1 ;
      end ;
      delete ;
    end ;
    else if nsbind = . then do ;
     nsbind = (scoldcpm/ncoldcpm)/hotcpm ;
    end ;
run ;

proc sort data = cpm ; by tag coldconc rep ; run ;

data cpm ;
    set cpm ;
    format coldconc bhotM bcoldM free btotM E10. spbind tracer 6.4 ;
    tracer = coldcpm/hotcpm ;
    spbind = tracer * (1 - nsbind) ;
    free   = 1 - tracer ;
    boverf = spbind/free ;
    bhotM  = (2.25E-16/volume) * hotcpm * (1 - nsbind) ;
    bcoldM = coldconc * spbind ;
    btotM  = bhotM + bcoldM ;
run ;

proc summary data = cpm nway ;
    class tag coldconc ;
    id sample hotcpm volume nsbind ;
    var coldcpm tracer spbind free boverf bhotM bcoldM btotM ;
    output out = cpm_var (drop = _type_ _freq_)
          mean = coldcpm tracer spbind free boverf bhotM bcoldM btotM
          std  = scoldcpm stracer sspbind sfree sboverf sbhotM sbcoldM sbtotM
          n(coldcpm) = n ;
run ;


data cpm_mean ;
    set cpm_var ;
    format coldconc bhotM bcoldM free btotM E10. spbind tracer 6.4 ;
    tracer = coldcpm/hotcpm ;
```

```
    spbind = tracer * (1 - nsbind) ;
    free   = 1 - tracer ;
    boverf = spbind/free ;
    bhotM  = (2.25E-16/volume) * hotcpm * (1 - nsbind) ;
    bcoldM = coldconc * spbind ;
    btotM  = bhotM + bcoldM ;
run ;

data _null_ ;
    file "&file..inp" notitle noprint ;
    set cpm_mean ; by tag ;
    if first.tag then put tag ;
    put (btotM boverf) (E12.);
    if last.tag then put " " ;
run ;

proc printto print = "&file..out" new ; run ;
proc print data = cpm_mean ;
  var coldconc coldcpm tracer spbind free boverf bhotM bcoldM btotM ;
  by tag hotcpm volume nsbind ;
run ;
proc printto ; run ;

proc printto print = "&file..var" new ; run ;
proc reg data = cpm_mean ;
  model sboverf = boverf / noint ;
run ;
proc printto ; run ;

/* Get output device */
%do %until(&dno. = 1 or &dno. = 2) ;
   %if &dno. ^=  %then %let sysmsg = Please enter 1 or 2 only. ;
   %display device bell ;
%end ;

%if &dno. = 1 %then %let pdevice = &screen. ;
%if &dno. = 2 %then %let pdevice = hplj300 ;
goptions device = &pdevice. ftext=swissl ;

%if &dno. = 2 %then x 'capture q=agl_artists_lzr timeout=0 nb nt nff' ;;
proc gplot data = cpm_var ;
    symbol1 v=dot i=rl0 w=3 ;
    plot sboverf*boverf ;
run ;
%if &dno. = 2 %then x 'endcap';;

%if &dno. = 2 %then x 'capture q=agl_artists_lzr timeout=0 nb nt nff' ;;
proc gplot data = cpm_var ;
    where coldconc > 0 and spbind ^= .  and coldconc ^= . ;
```

```
    symbol1 v=dot i=sm70 w=3 ;
    axis1 label=('Concentration M') logbase=10 ;
    axis2 minor=none label=('Percent Specific Bound ') ;
    plot spbind*coldconc / haxis = axis1 vaxis = axis2 ;
    by tag ;
run ;
%if &dno. = 2 %then x 'endcap';;
goptions device = &screen. ;
%mend cpm ;

%macro runcpm ;
%display file bell ;
%cpm(&fname.) ;
%mend runcpm ;

%runcpm ;
```

Program SCATCHXY for the fitting of Scatchard curves using the GAUSS package

```
/* Program to fit Scatchard curves to data, allowing errors in X and Y    **
** directions. Relative errors in X and Y must be specified.              **

** Copyright: D.B. Baird, AgResearch PO Box 60 Lincoln, New Zealand. 1992 **
** Unauthorized use prohibited                                            **
** Written in GAUSS 386i, Version 3.1, C. Aptech Systems, Inc.            **
**                  23804 SE Kent-Kangley Road, Maple Valley,             **
**                  Washington 98038, USA.                          */

#lineson ;
library pgraph,maxlik ;
declare matrix _u      != 0 ;
declare matrix _ratio  != 0 ;
declare matrix _errors != 0 ;
declare matrix _nplot  != 99 ;

proc ptrans(ab) ;
    local a,b,c,d,p,q,u,v ;
    a = ab[1] ; b = ab[2] ; c = ab[3] ; d = ab[4] ;
    p = a ;
    q = ln(c) ;
    u = a./b ;
    v = ln(c./d) ;
    retp(u|p|v|q) ;
endp ;

proc 6 = abtrans(p,ymax,smax) ;
    local a,b,c,d,u,v ;
    if rows(p) == 4 ;
      u = -p[1].*smax ;
      a =  p[2].*ymax ;
      v = -exp(p[3]).*smax ;
      c =  exp(p[4]).*ymax ;
      b = -a./u ;
      d = -c./v ;
    else ;
      u = 0 ;
      a =  p[1].*ymax ;
      v = -exp(p[2]).*smax ;
      c =  exp(p[3]).*ymax ;
      b = miss(0,0) ;
```

```
        d = -c./v ;
    endif ;
    retp(a,b,c,d,u,v) ;
endp ;

proc 4 = iptrans(p) ;
    local a,c,u,v ;
    if rows(p) == 4 ;
        u = p[1] ;
        a = p[2] ;
        v = exp(p[3]) ;
        c = exp(p[4]) ;
    else ;
        u = _u ;
        a = p[1] ;
        v = exp(p[2]) ;
        c = exp(p[3]) ;
    endif ;
    retp(u,a,v,c) ;
endp ;

proc intercpt(dxy) ;
    local x,y,s,a,b ;
    x = dxy[.,1] ;
    y = dxy[.,2] ;
    s = (y[2] - y[1])./(x[2] - x[1]) ;
    if s < 0 ;
        a = y[1] - x[1].*s ;
        b = x[1] - y[1]./s ;
    else ;
        a = maxc(vec(y)) ;
        b = maxc(vec(x)) ;
    endif ;
    s = a./b ;
    retp(a|b) ;
endp ;

proc pstart(dxy) ;
    local n ; n = rows(dxy) ;
    retp(intercpt(dxy[(n-1):n,.]) | intercpt(dxy[1:2,.])) ;
endp ;

proc fitymod(p,x) ;
    local tx,ac,ca,u,v,a,c,sq,y,pos ;
    {u,a,v,c} = iptrans(p) ;
    ca = c - a ;
    ac = a + c ;
    tx = (v - u).*x ;
    sq = tx.*(tx - 2.*ca) + ac^2 ;
```

```
    if sumc(sq .< 0) > 0 ;
      if _errors ;
        errorlog " Warning: FITYMOD - infeasable parameters for model" ;
      endif ;
      ndpclex ;
      retp(zeros(rows(x),1)) ;
    else ;
      retp((sqrt(sq) - (u+v).*x + ac)./2) ;
    endif ;
endp ;

proc fitxmod(p,y) ;
    local ty,n,u,v,a,c,bd,yf,pos ;
    {u,a,v,c} = iptrans(p) ;
    if scalmiss(_u) ;
      ty = (u-v).*y ;
      bd = a.*v + c.*u ;
      yf = (sqrt(ty^2+2.*ty.*(a.*v-c.*u)+bd^2) - (u+v).*y + bd)./(2*u*v) ;
    else ;
      n = rows(y) ;
      pos = selif(seqa(1,1,n),y.>a) ;
      yf = -1 - 1e+10.*(y-a)^2 ;
      if not scalmiss(pos) ;
        yf[pos] = (y[pos].*(y[pos]-a-c))./(v.*(a - y[pos])) ;
      endif ;
      if rows(pos) < n and _errors ;
        errorlog " Warning: FITXMOD - infeasable parameters " ;
        ndpclex ;
      endif ;
    endif ;
    retp(yf) ;
endp ;

proc resy(p,dxy) ;
    retp(-(1 - fitymod(p,dxy[.,1])./dxy[.,2])^2) ;
endp ;

proc resx(p,dxy) ;
    retp(-(dxy[.,1] - fitxmod(p,dxy[.,2]))^2) ;
endp ;

proc resxy(p,dxy) ;
    retp(_ratio.*resx(p,dxy) + (1 - _ratio).*resy(p,dxy)) ;
endp ;

proc res(p,dxy) ;
    if _ratio == 0 ;
      retp(resy(p,dxy)) ;
    elseif _ratio == 1 ;
```

```
        retp(resx(p,dxy)) ;
    else ;
        retp(resxy(p,dxy)) ;
    endif ;
endp ;

3proc dy(p,x) ;
    local dg,ac,ca,u,v,a,c,tx,sq ;
    x = x[.,1] ;
    dg = zeros(rows(x),4) ;
    {u,a,v,c} = iptrans(p) ;
    ca = c - a ;
    ac = a + c ;
    tx = (v - u).*x ;
    sq = sqrt(tx.*(tx - 2.*ca) + ac^2) ;
    if scalmiss(_u) ;
        dg[.,1] = -x.*((tx - ca)./sq + 1)./2 ;
    endif ;
    dg[.,2] = a.*((ac + tx)./sq + 1)./2 ;
    dg[.,3] = v.*x.*((tx - ca)./sq - 1)./2 ;
    dg[.,4] = c.*((ac - tx)./sq + 1)./2 ;
    retp(dg) ;
endp ;

proc du(p,u) ;
    retp(-1 ~ 0 ~ 0 ~ 0) ;
endp ;

proc da(p,a) ;
    retp(0 ~ 1 ~ 0 ~ 0) ;
endp ;

proc dv(p,v) ;
    retp(0 ~ 0 ~ -1 ~ 0) ;
endp ;

proc dc(p,c) ;
    retp(0 ~ 0 ~ 0 ~ 1) ;
endp ;

proc db(p,b) ;
    retp(-p[2]./p[1] ~ 1 ~ 0 ~ 0) ;
endp ;

proc dd(p,d) ;
    retp(0 ~ 0 ~ -1 ~ 1) ;
endp ;

proc rsscrit(&f,dxy,p,rss,df,lc,np) ;
```

```
    local f:proc ;
    retp(-sumc(f(p,dxy)) - rss.*(1 + lc.*np./df)) ;
endp ;

proc secant(&f,dxy,p,dp,rss,df,lc,np) ;
    local f:proc,p2,r1,r2,rn,fs,f1,f2,k,converge,tol ;
    clear converge ;
    tol = 1e-4 ;
    fs = rsscrit(&f,dxy,p,rss,df,lc,np) ;
    r1 = 0 ; f1 = fs ; r2 = 1 ; k = 0 ;
    do until(converge or k == 50) ;
      p2 = p + r2.*dp ;
      f2 = rsscrit(&f,dxy,p2,rss,df,lc,np) ;
      if scalmiss(f2) ;
        r2 = (r1+r2)./2 ;
      else ;
       if abs(f2 - f1) > tol^2 ;
          rn = (r1.*f2 - r2.*f1)./(f2 - f1) ;
       else ;
          rn = 2.*r2 - r1 ;
       endif ;
       if rn < 0 or abs(f2) > 10.*rss or f2 < (fs - tol) or iserr(f2) ;
          r2 = (r1+r2)./2 ;
       else ;
          r1 = r2 ;
          f1 = f2 ;
          r2 = rn ;
       endif ;
      endif ;
      converge = abs(r2-r1) < tol and abs(f2) < tol ;
      k = k + 1 ;
   endo ;
   if _errors ;
     if r2 > 100 ;
       errorlog "Warning: SECANT overflow"  ;
     elseif k == 100 ;
       errorlog "Warning: SECANT has not converged" ;
     endif ;
     format /rd 8,4 ;
     print "Secant ratio = " r2 ;
   endif ;
   retp(p+r2.*dp) ;
endp ;

proc ci(&dif,&f,dxy,p,V,rss,df,lc,np,xx,k) ;
    local dif:proc,f:proc,i,l,u,ol,ou,dg,dp,converge,tol,rms ;
    tol = 1e-4 ; clear converge ; rms = rss./df ;
    ol = p ; ou = p ;
    i = 1 ;
```

```
    do until(converge or i > k) ;
      dg = dif(ol,xx)' ;
      dp = -2.*sqrt(lc./(dg'V*dg))*(V*dg) ;
      l  = secant(&f,dxy,p,dp,rss,df,lc,np) ;
      dg = dif(ou,xx)' ;
      dp = 2.*sqrt(lc./(dg'V*dg))*(V*dg) ;
      u  = secant(&f,dxy,p,dp,rss,df,lc,np) ;
      converge = maxc(abs(l-ol|u-ou)) < tol ;
      ol = l ; ou = u ;
      i = i + 1 ;
    endo ;
    retp(l~u) ;
endp ;

proc 2 = fitconf(&f,dxy,p,V,rss,df,lc,np,x) ;
    local f:proc,n,l1,l2,fy,yl,yu,i,se,cV,cr,cc,k,xn,fc,gd ;
    n = rows(x) ;
    yl = zeros(n,1) ;
    yu = zeros(n,1) ;
    fy = fitymod(p,x) ;
    print "Fitting Confidence limits to curve:" ;;
    cr = csrlin ; cc = csrcol ;
    format /rd 5,1 ;
    i = 1 ;
    do until(i > n) ;
      k = 1 ;
      se = ci(&dy,&f,dxy,p,V,rss,df,lc,np,x[i],k) ;
      l1 = fitymod(se[.,1],x[i]) ;
      l2 = fitymod(se[.,2],x[i]) ;
      if l2 > fy[i] and fy[i] > l1 ;
        yl[i] = maxc(l1|0) ;
        yu[i] = l2 ;
      elseif fy[i] > l1 and fy[i] > l2 ;
        yl[i] = maxc(l1|0) ;
        cV = diagrv(zeros(rows(V),rows(V)),diag(V)) ;
        se = ci(&dy,&f,dxy,p,cV,rss,df,lc,np,x[i],k) ;
        l2 = fitymod(se[.,2],x[i]) ;
        if fy[i] > l2 ;
          l2 = fy[i] + (yu[i-1] - fy[i-1]) ;
        endif ;
        yu[i] = l2 ;
      elseif fy[i] < l1 and fy[i] < l2 ;
        yu[i] = l2 ;
        cV = diagrv(zeros(rows(V),rows(V)),diag(V)) ;
        se = ci(&dy,&f,dxy,p,cV,rss,df,lc,np,x[i],k) ;
        l1 = fitymod(se[.,1],x[i]) ;
        if fy[i] < l1 ;
          l1 = fy[i] + (yl[i-1] - fy[i-1]) ;
        endif ;
```

```
        yl[i] = l1 ;
      else ;
        yl[i] = maxc(l2 | 0) ;
        yu[i] = l1 ;
      endif ;
      locate cr,cc ; print (100.*i./n) "%" ;;
      i = i + 1 ;
    endo ;
    locate cr,1 ; print "                                                    " ;
    retp(yl,yu) ;
endp ;

/* Set up data */
start: trace 0 ; screen on ; cls ;
printdos "\27[33;47;1m" ;
print "Scatchard Plot Nonlinear Optimization Program (C. D Baird 1992)" ;
print " Using weights for y residuals proportional to 1/(y^2) " ;
printdos "\27[37;40;0m" ;
print ;
" Data file name (leave off .dat extension) = " ;; file = cons ; "\l" ;
/*
" Set X to Y error ratio (Y/N) ? " ;; xyratio = upper(cons) ; "\l" ;
*/ xyratio = "N" ;

if xyratio $== "Y" ;
  xyratio:
  print " A ratio of 0   = No error in X," ;
  print "           0.5 = Equal errors in X and Y," ;
  print "           1   = No error in Y\l" ;
  print " Give X to Y ratio for error variances (in interval [0,1]) = " ;;
  _ratio = con(1,1) ; " " ;
  if _ratio < 0 or _ratio > 1 ; goto xyratio ; endif ;
else ;
  _ratio = 0 ;
endif ;
" Fix the value of lower slope to be zero (Y/N) ? " ;; ans = cons ; "\l" ;
if upper(ans) $== "Y" ;
  _u = 0 ;
else ;
  _u = miss(0,0) ;
endif ;
infile  = file $+ ".dat" ;
outfile = file $+ ".out" ;
load data[] = ^infile ;
data = sortc(reshape(data,(rows(data)*cols(data))./2,2),1) ;
x = data[.,1] ;
y = data[.,2] ;
n = rows(x) ;
xmax = maxc(x) ;
```

```
xmin = minc(x) ;
ymax = maxc(y) ;
ymin = minc(y) ;
smax = ymax./xmax ;
dxy = (x./xmax) ~ (y./ymax) ;

/* Obtain starting parameter values */
p0 = ptrans(pstart(dxy)) ;

/* Optimise parameters */
maxset ;
_mlmiter = 100 ;
_mlcovp  = 3 ;
_mlstmth = "Newton golden" ;
_mlmdmth = "Newton golden" ;
_mlndmth = "" ;
__title = "Maximum Likelihood optimisation of Scatchard Curve" ;

if not scalmiss(_u) ;
   p0 = p0[2:4] ;
   let _mlparnm = "a" "ln(c/d)" "ln(c)" ;
else ;
   let _mlparnm = "a/b" "a" "ln(c/d)" "ln(c)" ;
endif ;

output file = ^outfile reset ;
fit: {parms,fmin,grd,cov,retc} = maxprt(maxlik(dxy,1|2,&resy,p0)) ;

if xyratio $== "Y" and _ratio /= 0 ;
   yparms = parms ;
   ycov   = cov ;
   yrss   = -fmin ;
   __title = "ML optimisation of Scatchard Curve using XY error ratio" ;
   _mlstmth = "bfgs golden" ;
   _mlmdmth = "Newton golden" ;
   parms[1] = maxc((minc(dxy[.,2]) - 1e-4)|0) ;
   {parms,fmin,grd,cov,retc} = maxprt(maxlik(dxy,1|2,&res,parms)) ;
endif ;

if scalmiss(_u) and parms[1] < 5e-3 ;
   print "\l The slope of flat line is tending to zero." ;;
   print " The model has become degenerate." ;
   print " Fitting the reduced model with slope = 0 \l" ;
   _u = 0 ;
   p0 = parms[2:4] ;
   let _mlparnm = "a" "ln(c/d)" "ln(c)" ;
   __title = "ML optimisation of Reduced Scatchard Curve" ;
   goto fit ;
endif ;
```

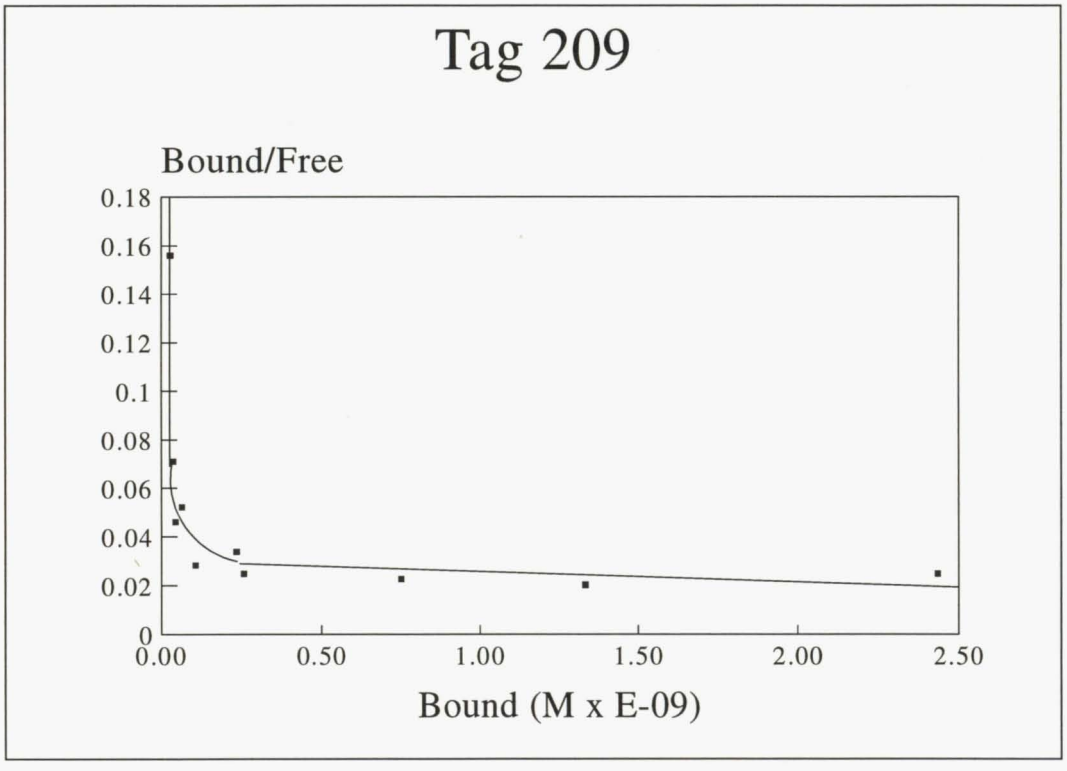
```
if iserr(cov) ; cov = miss(0,0) ; endif ;

/* Convert to defining parameters */
{a,b,c,d,u,v} = abtrans(parms,ymax,smax) ;

/* Fit confidence limits to parameters */
rss = -fmin.*n ;
np  = rows(p0) ;
df  = n - np ;
fy  = ymax.*fitymod(parms,dxy[.,1]) ;

if not scalmiss(_u) ;
  parms = _u | parms[1:3] ;
  if not ismiss(cov) ;
    newc = zeros(4,4) ;
    newc[2:4,2:4] = cov ;
    cov = newc ;
    clear newc ;
  endif ;
endif ;

if not ismiss(cov) ;
  lc = invcdff(.95,np,df) ;
  al = ci(&da,&res,dxy,parms,cov,rss,df,lc,np,0,20) ;
  al = ymax.*sortc(vecr(al[2,.]),1) ;
  al[1] = maxc(al[1]|0) ;
  cl = ci(&dc,&res,dxy,parms,cov,rss,df,lc,np,0,20) ;
  cl = ymax.*sortc(vecr(exp(cl[4,.])),1) ;
  cl[1] = maxc(cl[1]|0) ;
  vl = ci(&dv,&res,dxy,parms,cov,rss,df,lc,np,0,20) ;
  vl = smax.*sortc(vecr(-exp(vl[3,.])),1) ;
  if not scalmiss(_u) ;
    b = miss(0,0) ; bl = miss(0|0,0) ;
    u = 0 ; ul = zeros(2,1) ;
  else ;
    ul = ci(&du,&res,dxy,parms,cov,rss,df,lc,np,0,20) ;
    ul = smax.*sortc(vecr(-ul[1,.]),1) ;
    bl = ci(&db,&resy,dxy,parms,cov,rss,df,lc,np,0,40) ;
    bl = xmax.*sortc(vecr(bl[2,.]./bl[1,.]),1);
    if ul[2] > 0 ;
      bl[1] = minc(bl) ;
      bl[2] = 0v7ff0000000000000 ;
    endif ;
  endif ;
  dl = ci(&dd,&res,dxy,parms,cov,rss,df,lc,np,0,40) ;
  dl = xmax.*sortc(vecr(exp(dl[4,.]-dl[3,.])),1) ;
else ;
  al = miss(0|0,0) ;
  bl = al ; cl = al ; dl = al ; ul = al ; vl = al ;
```

```
endif ;

/* Print out Results */
format /rz 10,4 ;
print "   ------ Data ------    ----- Curve -----"  ;
print "      x         y        Fitted     Residual" ;
print x~y~fy~(y-fy) ;
print "\l\lFitted parameters\l" ;
print "Residual SS = " rss " with " df "df\l" ;
if _ratio > 0 ;
  print "Using an X-Y error ratio of " _ratio "\l" ;
endif ;
print "Flat line:" ;
print "Slope       = " u "   95% CI = ( " ul[1] ", " ul[2] ")" ;
print "Y intercept = " a "   95% CI = ( " al[1] ", " al[2] ")" ;
print "X intercept = " b "   95% CI = ( " bl[1] ", " bl[2] ")\l" ;
print "Steep line:" ;
print "Slope       = " v "   95% CI = ( " vl[1] ", " vl[2] ")" ;
print "Y intercept = " c "   95% CI = ( " cl[1] ", " cl[2] ")" ;
print "X intercept = " d "   95% CI = ( " dl[1] ", " dl[2] ")\l" ;
output off ;

/* Plot graph */
graphset ;
px = seqa(xmin,(xmax-xmin)./_nplot,_nplot+1) ;
py = ymax.*fitymod(parms,px./xmax) ;
if not ismiss(cov) ;
  {yl,yu} = fitconf(&res,dxy,parms,cov,rss,df,lc,np,px./xmax) ;
  yl = ymax.*yl ;
  yu = ymax.*yu ;
  scale(0|x|px,0|y|py|yl|yu) ;
else ;
  print "Confidence limits could not be fitted" ;
  scale(0|x|px,0|y|py) ;
endif ;

x11 = _pworld[1] ;
x21 = _pworld[1] ;
y11 = a + u.*x11 ;
y21 = c + v.*x21 ;
y12 = _pworld[3] ;
y22 = _pworld[3] ;
x12 = (y12 - a)./u ;
x22 = (y22 - c)./v ;
if x12 > _pworld[2] or u == 0 ;
  x12 = _pworld[2] ;
  y12 = a + u.*x12 ;
endif ;
if x22 > _pworld[2] or v == 0 ;
```

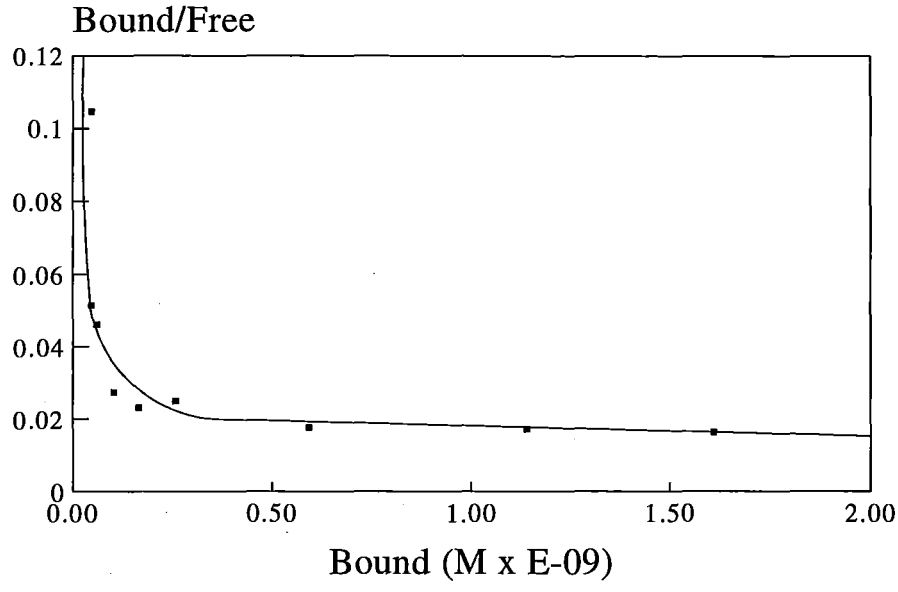
```
   x22 = _pworld[2] ;
   y22 = c + v.*x22 ;
endif ;
_pline = (1~6~x11~y11~x12~y12~0~9~2) |
         (1~6~x21~y21~x22~y22~0~9~2) ;
clearg _pdate ;
_pnum = 2 ;
_pcrop  = 1 ~1 ~1 ~1 ~0 ;
_psym   = x ~ y ~ ((8~5~12~1~2).*ones(n,1)) ;
_plctrl = 0 ;
_pltype =  6 ~  3 ~  3 ;
_pcolor = 14 ~ 12 ~ 12 ;
_plwidth = 4 ~  2 ~  2 ;
_ptek = file $+ ".tkf" ;
fonts("microb") ;
xlabel("Total Bound (M)");
ylabel("Bound/Free") ;
title("Tag " $+ upper(strsect(file,2,strlen(file)))) ;
if not ismiss(cov) ;
   xy(px,py~yl~yu) ;
else ;
   xy(px,py) ;
endif ;

/* Produce HPG output */
print "Do you want to produce a HPG file to import into Wordperfect (Y/N)? " ;;
if (upper(cons) $/= "N") ;
   cls ;
   hardcopy("C3") ;
   _pcvfile = file $+ ".hpg" ;
   replay ;
endif ;
print ;

/* Rerun for new case */
print "Do you want to process another plot (Y/N)? " ;;
if (upper(cons) $== "Y") ; goto start ; endif ;
cls ;
ndpclex ;
end ;
```

Scatchard curves for insulin binding to particulate membrane fraction of ovine skin

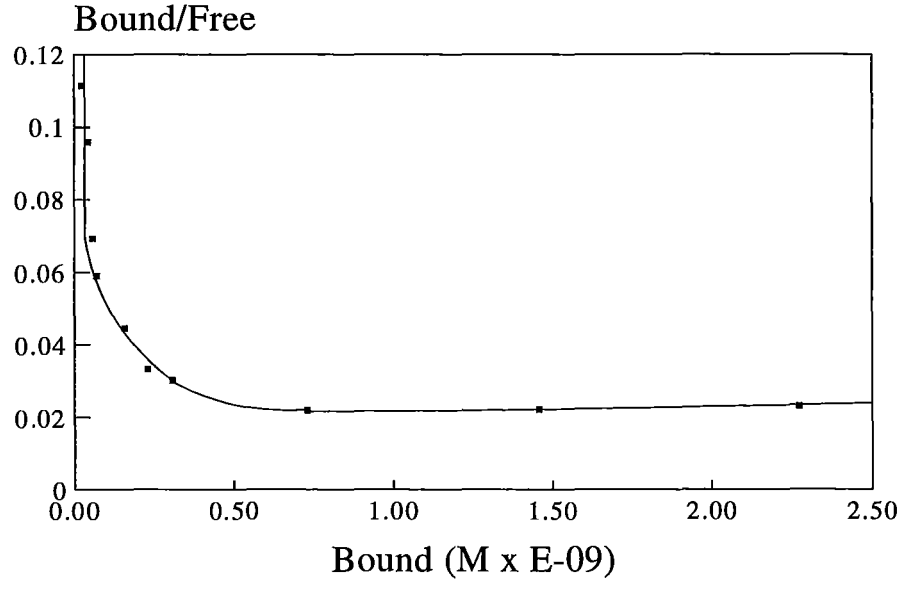
The Scatchard plots for the animals not presented in Chapter 6 follow. Curves have been fitted by eye for the purpose of this presentation. Computer fitted curves were used to generate the two lines from which receptor capacity and affinity were obtained (see section 6.2.4.2).



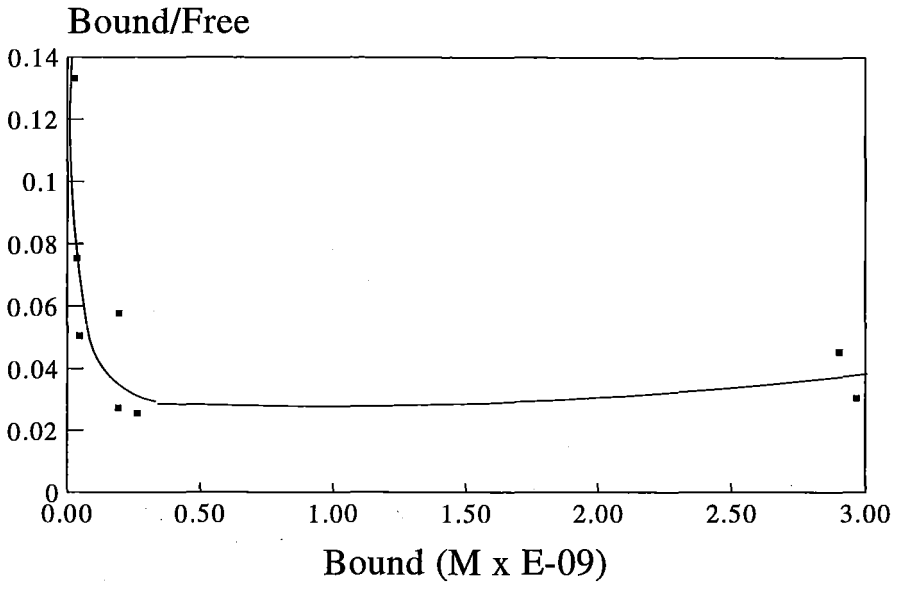
Tag 439



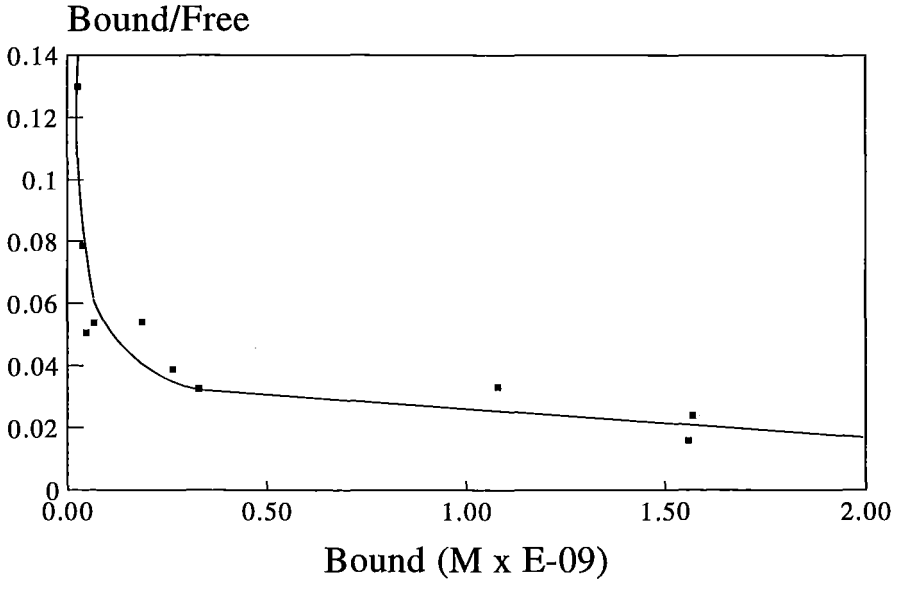
Tag 440



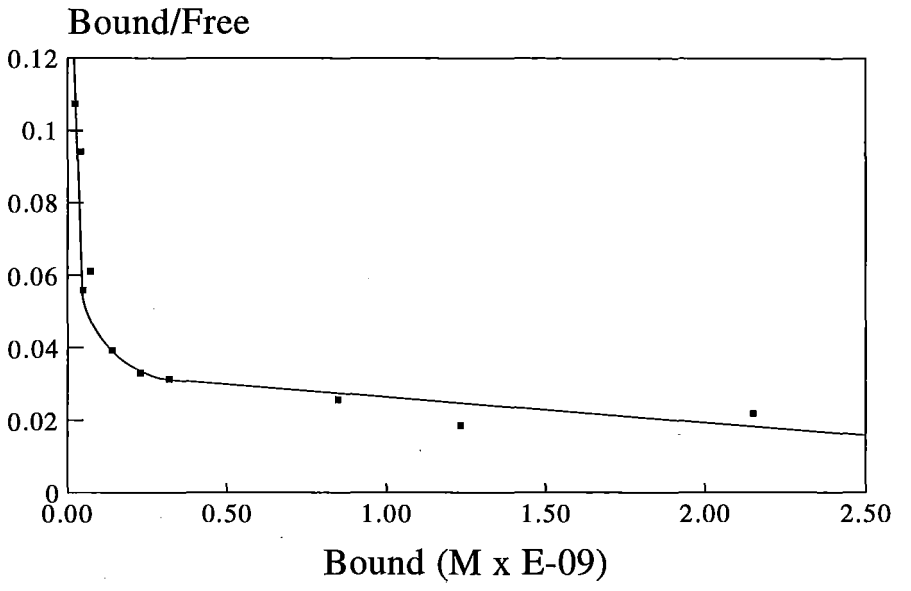
Tag 641



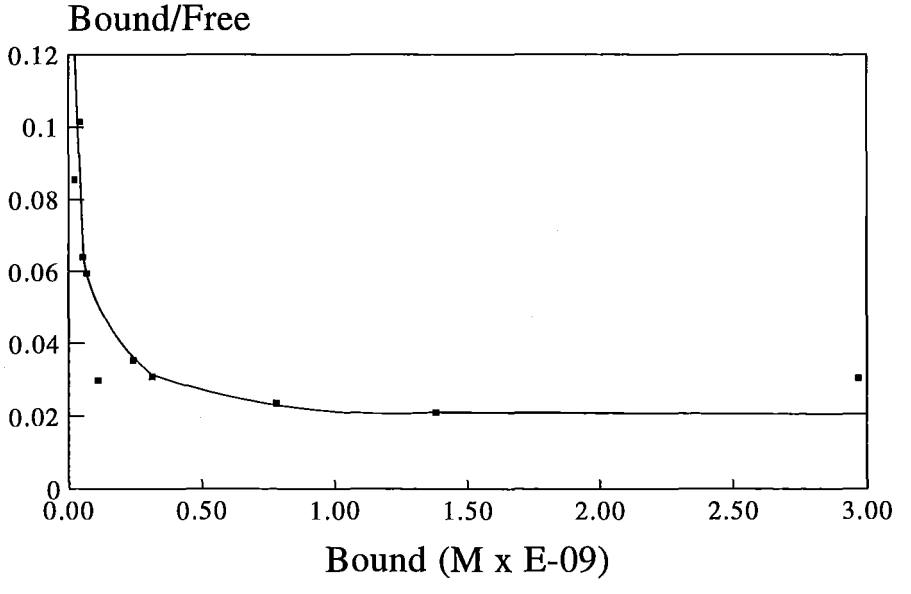
Tag 604



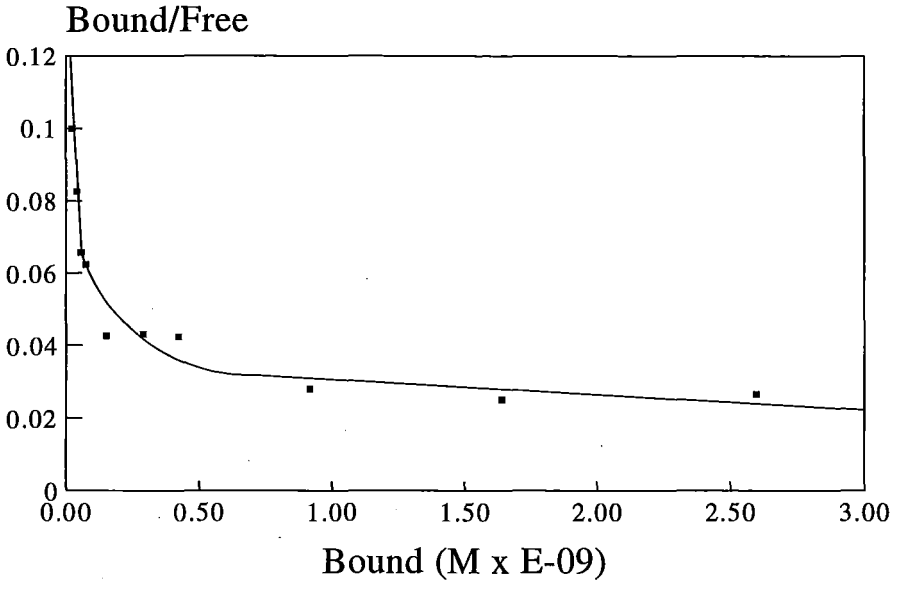
Tag 617



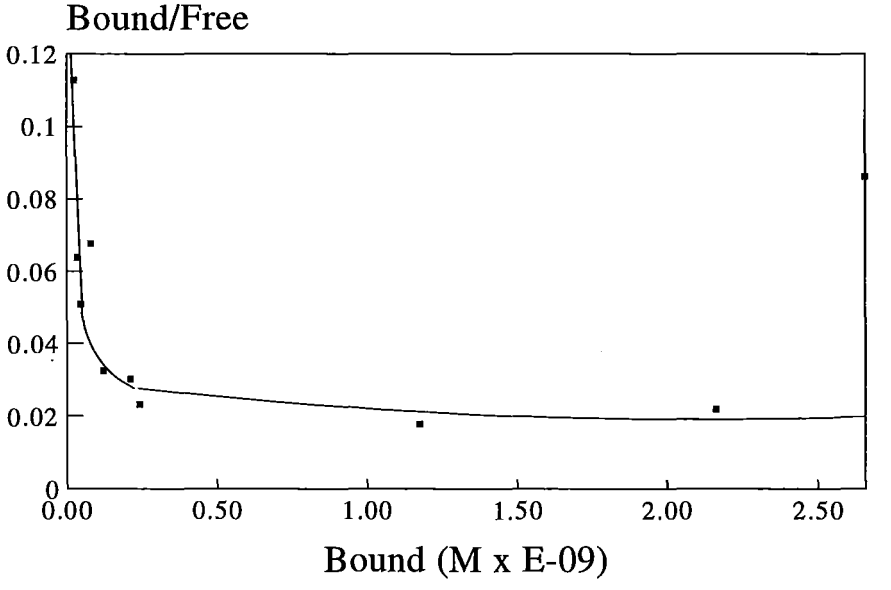
Tag 618



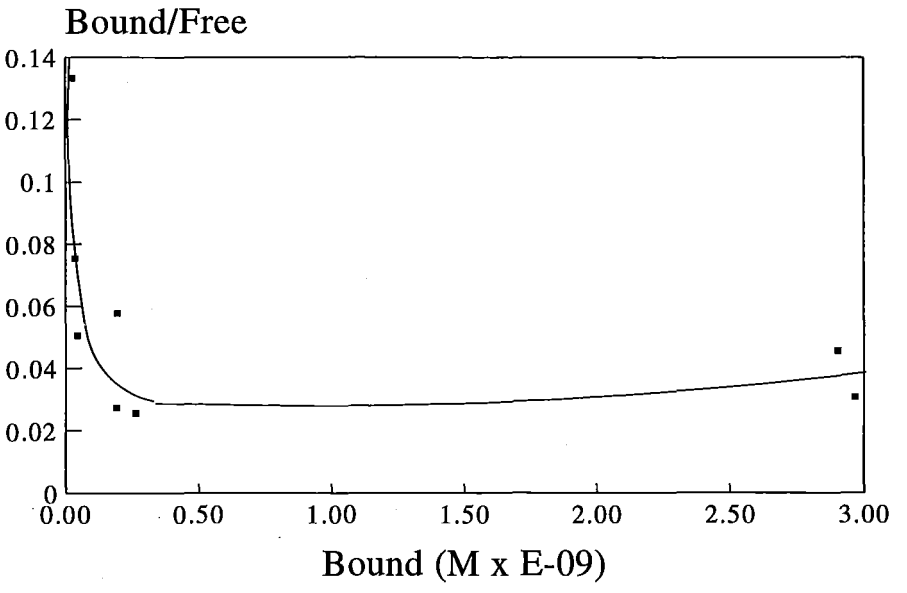
Tag 623



Tag 640



Tag 641


Bound/Free
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0.00
0.50
1.00
1.50
2.00
2.50
3.00
Bound (M x E-09)

Frequently used solutions and media

Adelaide's B buffer (for 100 ml)

10 mM Tris-HCl pH 7.5	(1 ml of 1 M stock)
10 mM NaCl	(1 ml of 1 M stock)
50 mM Na_4EDTA	(20 ml of 0.25 M stock)

100 X Denhardts solution (for 100 ml)

2 % BSA	(2 g)
2 % Ficoll	(2 g)
2 % polyvinylpyrrolidone	(2 g)

Deionized formamide

Formamide (analytical grade - Sigma) was deionised by mixing with Biorad AG-501 X8 resin (50 g/500 ml) until the dye changes colour. Formamide was filtered through filter paper before storage at -20^{0}C.

2 x Freezing medium (for 1 litre)

7.2 mM K_2HPO_4
2.6 mM KH_2PO_4
4.0 mM NaCitrate
1.0 mM $MgSO_4.7H_2O$
Glycerol 90 ml

Luria-Bertani (LB) medium pH 7.4 (for 1 litre)

NaCl 10 g
Yeast Extract 5 g
Bactotryptone 10 g

For plates add 15 g agar

PES solution (for 100 ml)

5 M Potassium acetate	(29.44 g)
Glacial acetic acid	(11.5 ml)

PRS (for 1 litre)

10 % sucrose	(100 g)
50 mM Tris-HCl pH 8.0	(50 mls of 1 M stock)

Phosphate buffered saline (PBS/50mM EDTA, for 1 litre)

10 Dulbecco A tablets
5 ml Dulbecco B mineral salt solution
18.612 g Na_2EDTA

Red blood cell (RBC) lysing solution (for 1 litre)

150 mM NH_4Cl	(8.02g)
10 mM $KHCO_3$	(1.00g)
0.1 mM Na_2EDTA	(400µl of 0.25M stock)

Solution D

guanidium thiocyanate	(250 g)
dd H_20	(319.4 ml)
NaCitrate	(17.6 ml 0.75 M stock pH 7)

Before use add 0.36 µl/50 ml stock of filtered mercaptoethanol.

SOC pH 7 (for 25 mls)

Bactotryptone	(0.5 g)
Yeast	(0.125 g)
NaCl	(0.063 g)
KCl	(0.25 ml of 250 mM stock)
$MgCl_2$	(125 µl of 1 M stock)
Glucose	(500 µl of 1 M stock)

Solution II (for 10 mls)

0.2 M NaOH	(400 µl of 5 M stock)
1% SDS	(1 ml 10 % stock)

20 x SSC pH 7.0 (for 1 litre)

NaCl	(175.3 g)
NaCitrate	(88.2 g)

20 X SSPE pH 7.4 (for 1 litre)

NaCl	(174.0 g)
$NaH_2PO_4.H_20$	(27.6 g)
Na_4EDTA	(7.4 g)

STE

10 mM Tris-HCl pH 8.0	(10 mls of 1 M stock)
100 mM NaCl	(20 mls of 5 M stock)
1 mM Na_2EDTA	(9.3 g)

10 X TBE

0.89 M Tris	(108 g)
0.89 M Boric Acid	(55 g)
0.02 M Na_2EDTA	(9.3 g)

50 X TE

20.5 M Tris-HCl pH 8.0	(500 ml of 1M stock)
0.05 M Na_2EDTA	(200 ml of 0.25M stock)

TBS pH 7 (for 1 litre)

140 mM NaCl	(8.00 g)
0.5 mM KCl	(0.38 g)
24 mM Tris	(3.00 g)
Phenol red	(0.015 g)

Terrific Broth + ampicillin

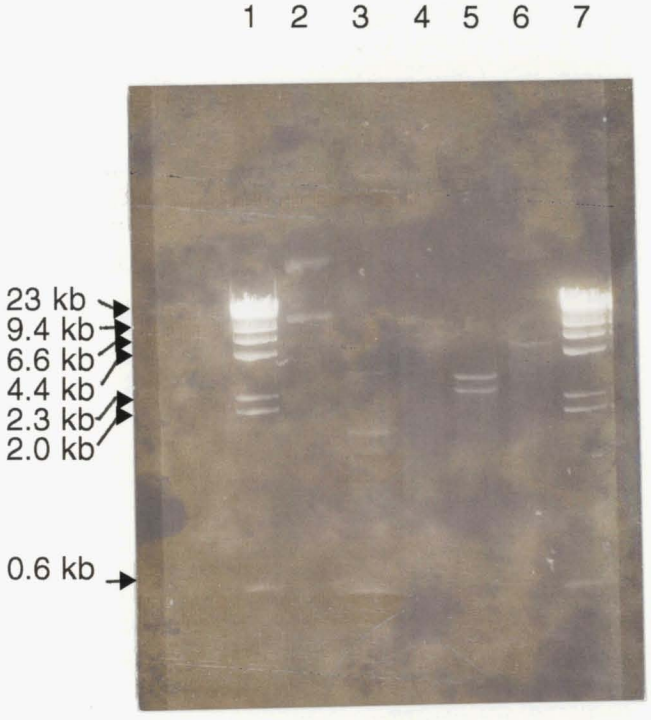
To 900 ml H_2O add:

Bactotryptone	(12 g)
Bacto-yeast	(24 g)
Glycerol	(4 ml)

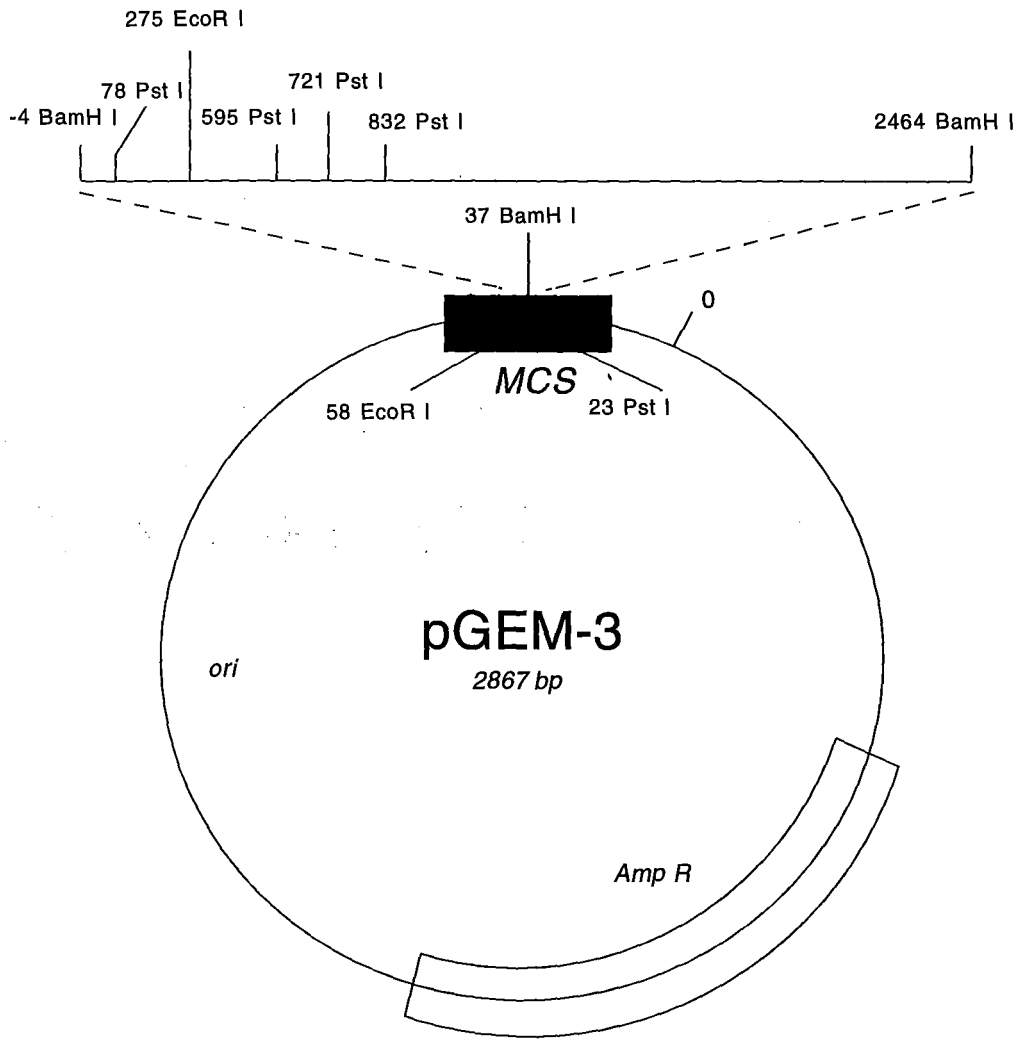
Dissolve and autoclave for 20 minutes at 15 lb/sq.in.

Cool to 60 °C and add 100 ml of a sterile solution of 0.17 M KH_2PO_4 0.72 M K_2HPO_4.

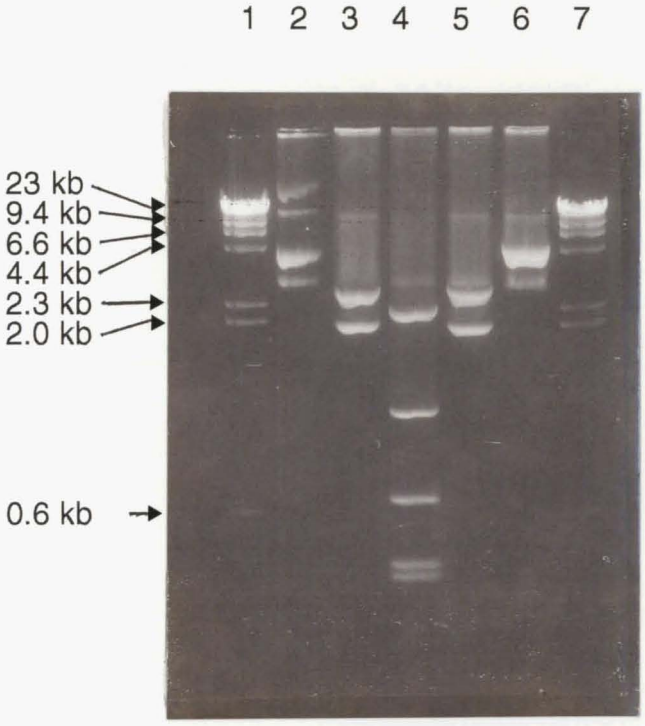
Plasmid maps and plasmid verification



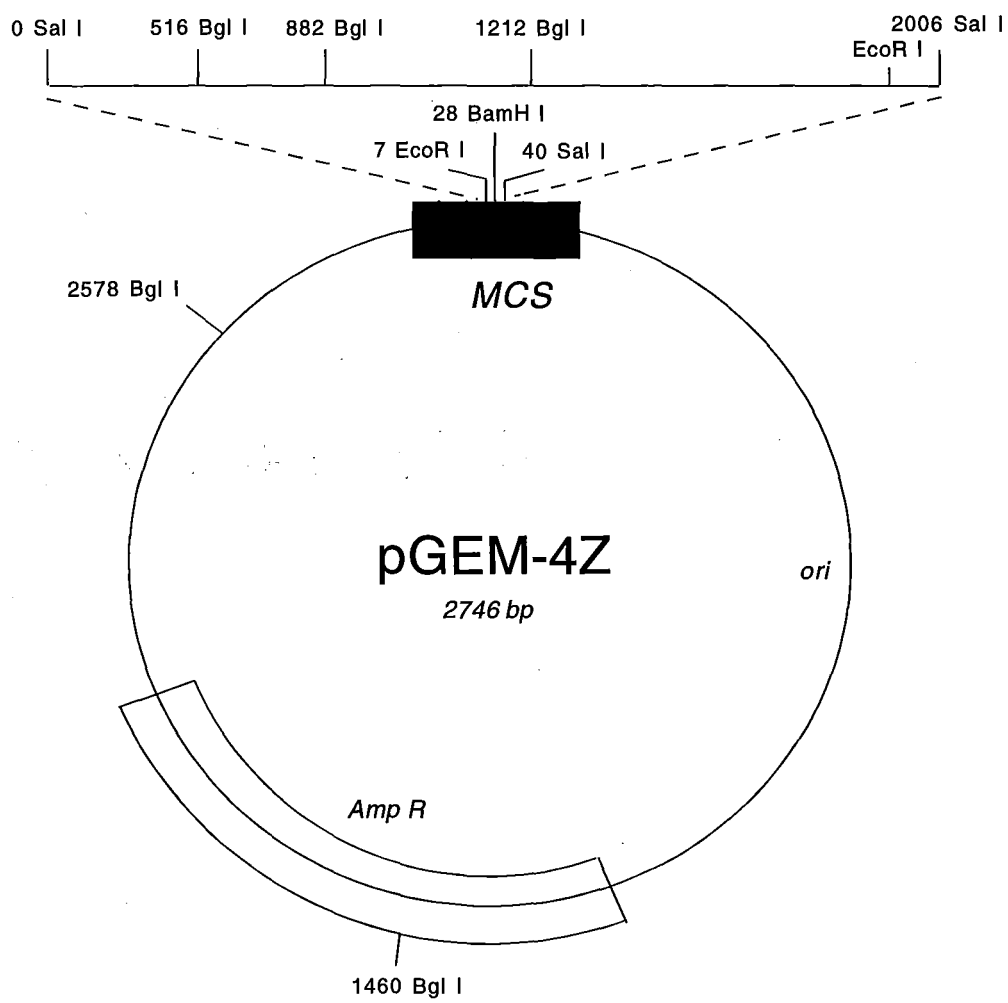
Restriction digestion of the pGLUT1 plasmid. Lane 1) Lambda Hind III digest 2) uncut plasmid 3) Pst I digest 4) EcoR I digest 5) BamH I digest 6) Xba I digest 7) Lambda Hind III digest.



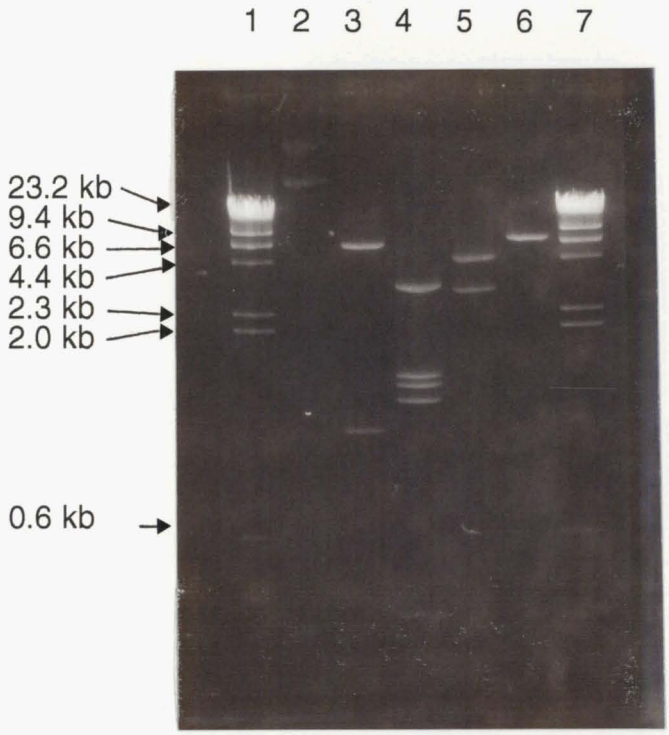
Human glucose transporter 1 cDNA, (2468 bp) inserted into the vector pGEM-3. Total plasmid size 5335 bp.



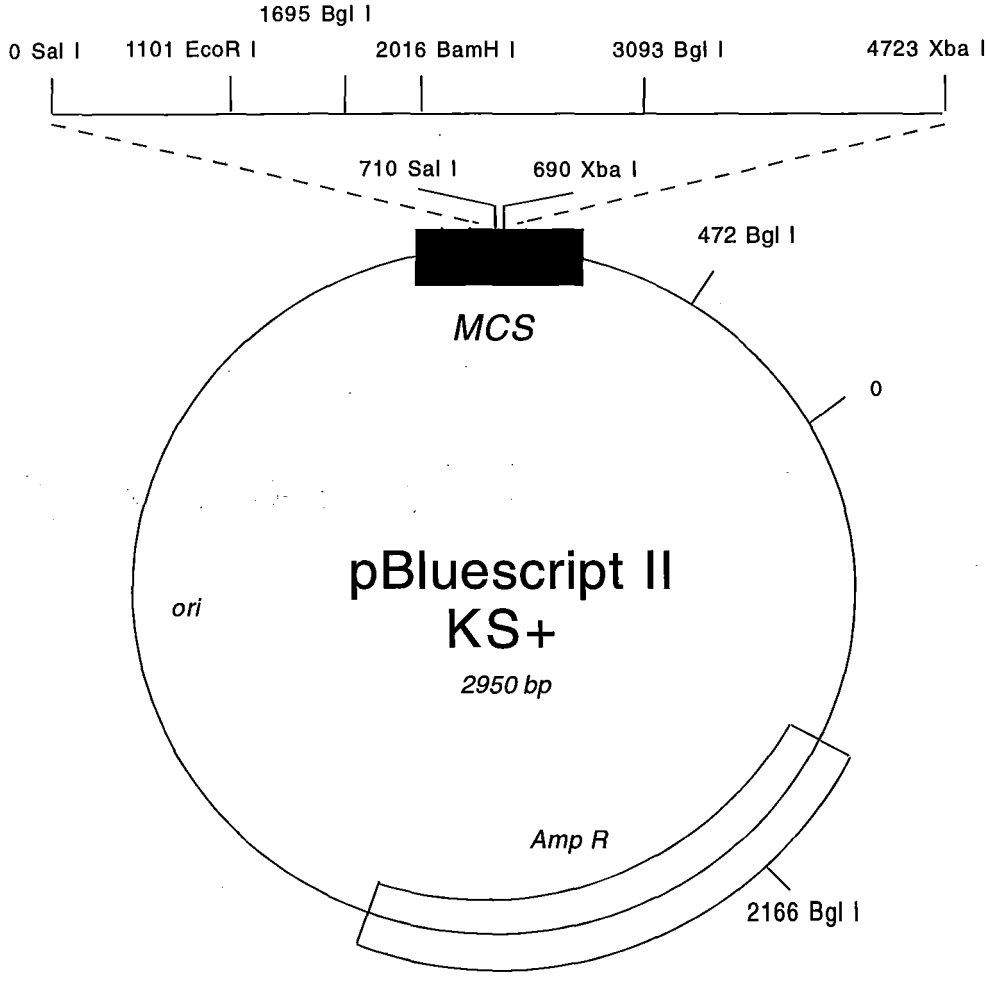
Restriction digestion of the pGLUT4 plasmid. Lane 1) Lambda Hind III digest 2) uncut plasmid 3) EcoR I digest 4) Bgl I digest 5) Sal I digest 6) BamH I digest 7) Lambda Hind III digest.



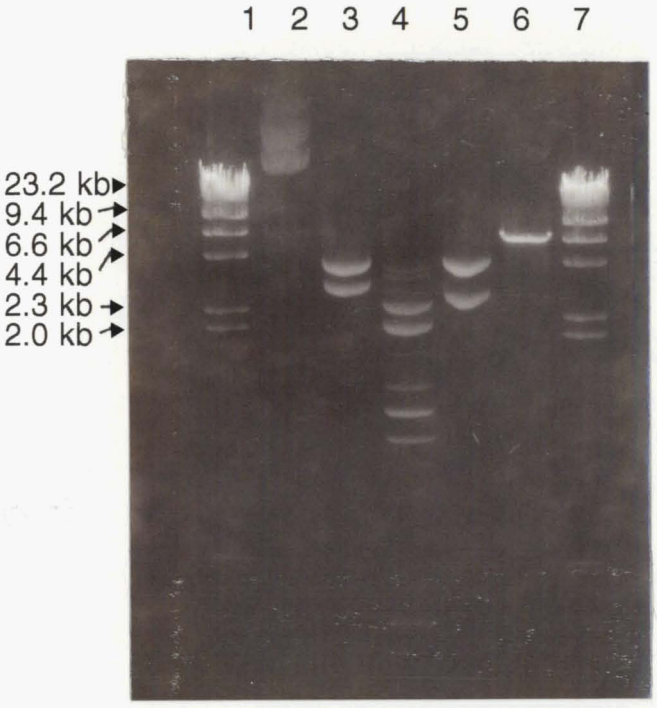
Human glucose transporter 4 cDNA (2006 bp), inserted into the vector pGEM-4Z. Total plasmid length 4752 bp.



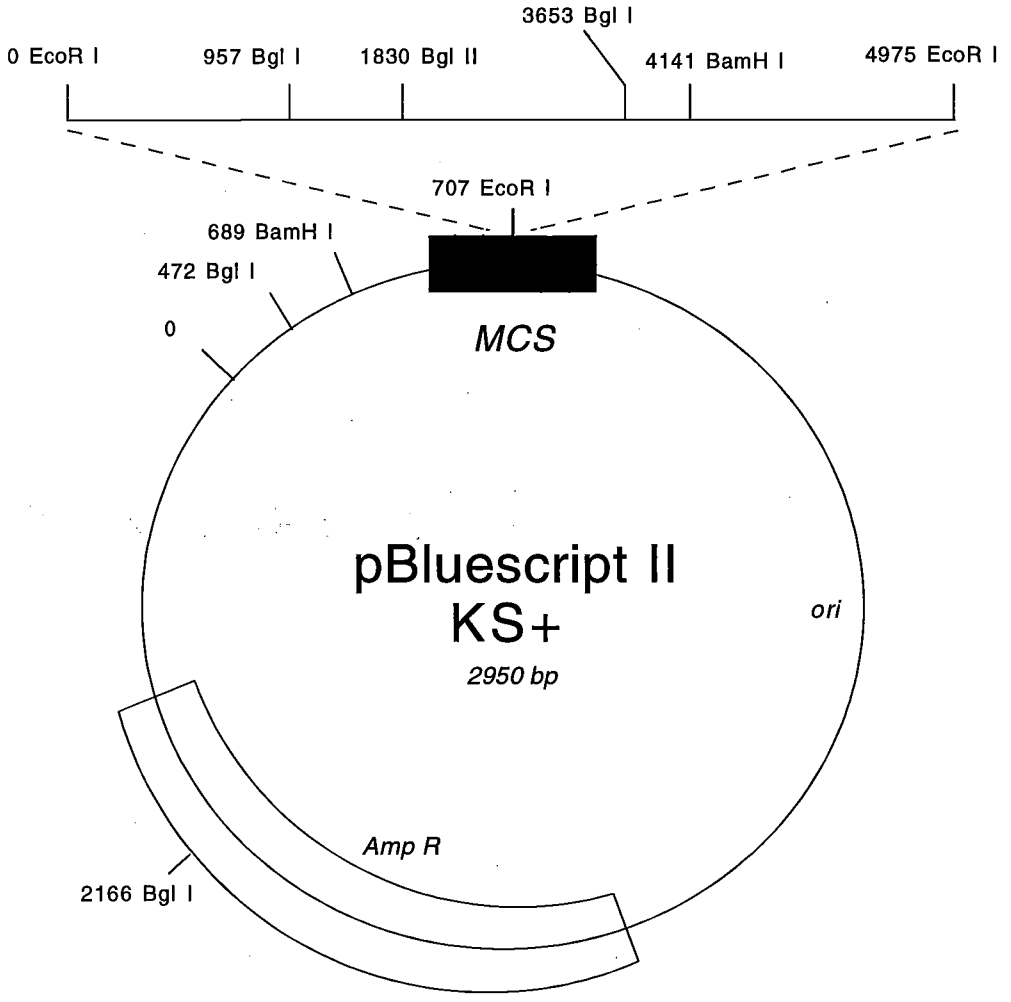
Restriction digestion of the pIR plasmid. Lane 1) Lambda Hind III digest 2) Uncut plasmid 3) EcoR I digest 4) Bgl I digest 5) Xba I/Sal I digest 6) BamH I digest 7) Lambda Hind III digest.



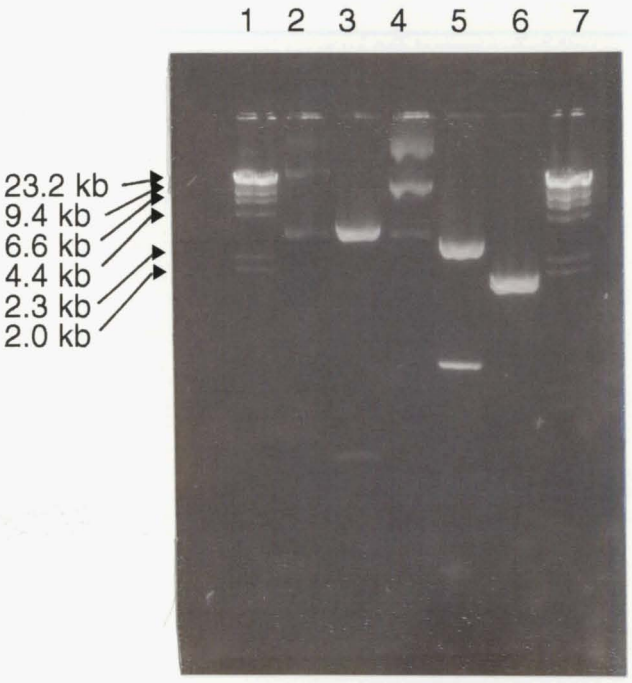
Human insulin receptor cDNA (4723bp), inserted into the vector pBluescript KS+. Total plasmid size 7673 bp.



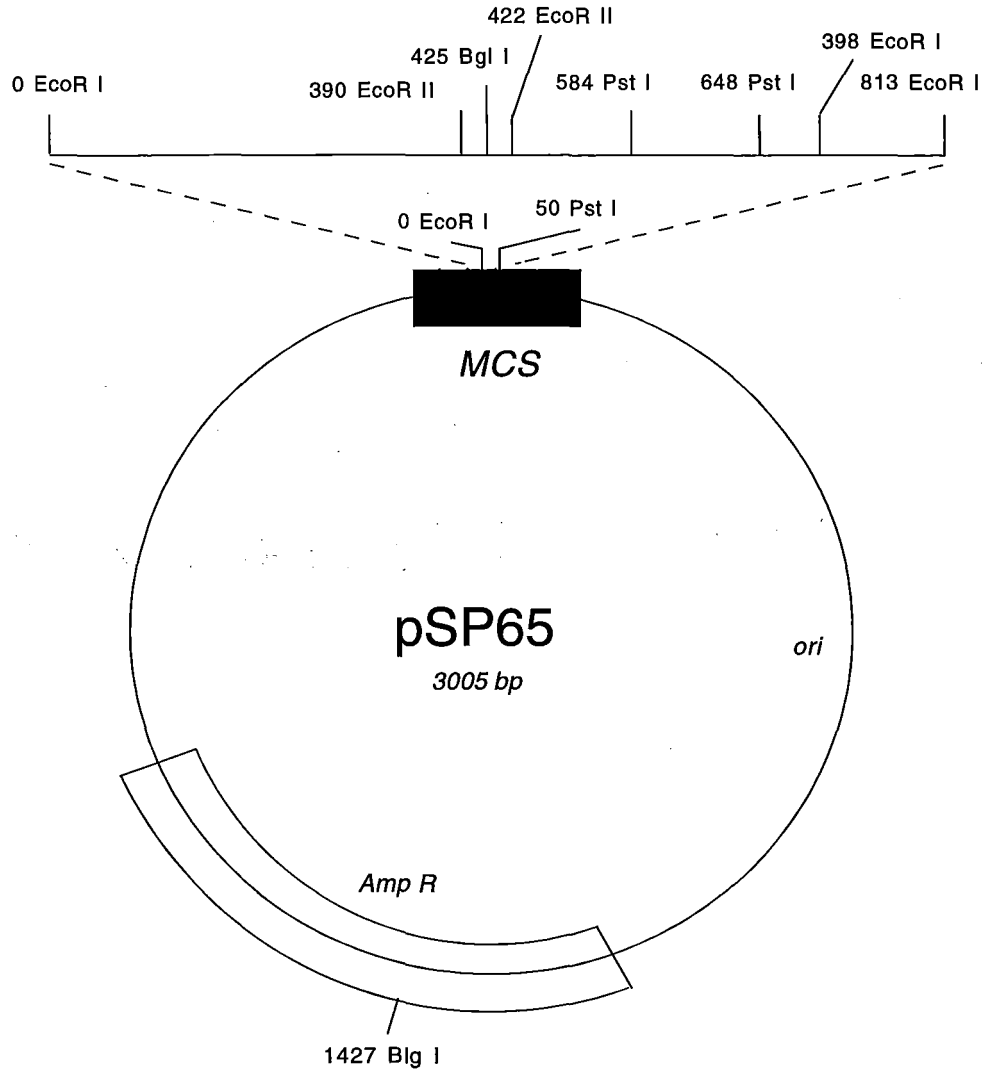
Restriction digestion of the pIGF-IR plasmid. Lane 1) Lambda Hind III digest 2) uncut plasmid 3) BamH I digest 4) Bgl I digest 5) EcoR I digest 6) Bgl II digest 7) Lambda Hind III digest.



Human insulin-like growth factor I cDNA (4975 bp), inserted into the vector pBluescript KS+. Total plasmid size 7925 bp.



Restriction digestion of the pRPS6 plasmid. Lane 1) Lambda Hind III digest 2) Uncut plasmid 3) Pst I digest 4) EcoR II digest 5) EcoR I digest 6) Bgl I digest 7) Lambda Hind III digest.



Human ribosomal protein S6 cDNA (813 bp), inserted into the vector pSP65. Total plasmid size 3818 bp.

RNA extraction procedure - acid guanidium thicyanate method

This method was adapted from Chomczynski and Scacchi (1987).

1. Pieces of tissue of approximately 150 mg (wet weight), were collected into 700 µl solution D or alternatively scraped into liquid nitrogen using a sterile scalpel blade (for the wool follicles). The tissue was homogenized using a polytron, for 15 seconds intervals at half speed.

2. The following addition were made each followed by a brief mix by inversion.

   70 µl 2 M NaAcetate pH 4 (DEPC treated)
   700 µl phenol
   140 µl chloroform-isoamyl (24:1)

3. The tubes were shaken for 10 s and placed on ice for 15 minutes.

4. The cellular debris was pelleted by a 12000 rpm spin at 4 ^{0}C for 20 minutes.

5. 700 µl of the aqueous layer was transferred to a new tube and total RNA precipitated with an equal volume of isopropanol overnight at -20 0C.

6. The precipitate was spun down by a 12000 rpm spin at 4 ^{0}C for 20 minutes.

7. The pellet was resuspended in 0.3 ml solution D.

8. RNA precipitated a second time as before. Samples were put away for long-term storage at this point.

9. Pellets were spun down as in step 6 and resuspended in 100 µl of DEPC treated water.

10. The quality of the RNA yield was assessed by agarose gel electrophoresis. When quality was insufficient RNA was subjected to an ethanol precipitation and a 70% ethanol wash before resuspension in DEPC treated water.

11. Yields were estimated by spectrophotometry by diluting a 3 µl sample in 747 µl of DEPC treated water. 260:280 ratios varied between 1.3 and 1.6 and yields from 200-1200 µg depending on the tissue.

Transformation of competent DH5α cells

1. 2 µl of a thawed DH5α stock was incubated overnight in a 25 ml aliquot of LB broth at 37 ^{0}C, with vigorous shaking.

2. The next morning a 50 µl aliquot was taken and innoculated into a fresh flask containing 25 mls of LB broth.

3. The flask was again incubated at 37 ^{0}C with vigorous shaking, and after 3 hours the OD_{600} was checked against a reference tube of LB broth at quarter hourly intervals. When the OD reached 0.39-0.45, the culture was transferred to two chilled sterile 10 ml Falcon tubes.

4. Tubes were cooled on ice for 30 minutes and then spun at 3 500 rpm for 10 minutes at 4 ^{0}C.

5. The supernatant was decanted and tubes left to drain for 1 minute. Pellets were resuspended in 2.5 ml of ice-cold 10 mM $CaCl_2$/10% glycerol.

6. Cells were cooled on ice for 30 minutes and then pelleted by centrifugation for 10 minutes at 2 500 rpm at 4^{0}C.

7. The supernatant was decanted and the cells were resuspended GENTLY in 0.5 mls of ice-cold 75 mM $CaCl_2$/10 % glycerol.

8. 200 µl aliquots of competent cells were transferred to Eppendorf tubes.

9. 50 ng of DNA in a maximum volume of 10 µl, was added to the competent cells. A control was also prepared by adding 10 µl of H_2O.

10. Tubes were swirled gently and stored on ice for 30 minutes.

11. Cells were heat shocked at 42 ^{0}C for 90 seconds.

12. After chilling on ice for 2 minutes 800 µl of SOC was added and tubes were incubated at 37 ^{0}C for 45 minutes.

13 The contents of each Eppendorf tube was spread out on LB + Amp plates with a glass sterile rod and incubated upside down at 37 ^{0}C overnight.

Mini preparation of plasmid DNA (alkaline lysis method)

1. 5 ml of LB media was innoculated with a single bacterial colony and inculbated over night at 37^{0}C with vigorous shaking.

2. 1.5 ml of culture was poured into an Eppendorf, spun for 1 minute at 12 000 rpm and the supernatant aspirated off.

3. The pellet was resuspended in 100 µl of ice cold PRS and stored at room temperature for 5 minutes.

4. 200 µl of freshly prepared solution II was added and tubes inverted three times to mix the contents. Tubes were stored on ice for 5 minutes.

5. 150 µl of PES was added and tubes were gently vortexed in an inverted position for 10 seconds. Tubes were stored on ice for 5 minutes.

6. The curd was pelleted by a 5 minute spin at 12 000 rpm and the supernatant transferred to a fresh tube.

7. The samples were extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (1:1). Tubes were inverted gently to mix in between steps and the layers separated by brief centrifugation. The aqueous layer was transferred each time to a fresh tube.

8. A 10 % volume of Na acetate was added.

9. Nucleic acid was precipitated with 2 volumes of ethanol and stored for 5 minutes.

10. Pellets were formed by centrifugation for 5 minutes and supernatant sucked off with a pipette tip.

11. Pellets were washed with a volume of 70 % ethanol, followed by a 5 minute spin.

12. The supernatant was aspirated off by pipette and pellets dried in a Speedivac.

13. The pellets were resuspended in 39 µl of TE containing 1 µl of DNase-free pancreatic RNase (20µg/ml).

Large scale preparation of plasmid DNA

1. One litre of terrific broth was innoculated with 200 µl of fresh culture or 400 ul of frozen cells. The culture was incubated with vigorous shaking for 14 hours in a 37 ^{0}C shaking incubator.

2. Cells were harvested by aliquoting the culture into 250 ml centrifuge bottles and spinning at 4000 rpm for 15 minutes at 4 ^{0}C in a Sorvall centrifuge (GSA rotor).

3. The cells were resuspended and washed in a 100 mls of ice cold STE.

4. Cells were collected as before.

5. The pellet was resuspended in 18 mls of PRS.

6. The following additions were made:

   2 mls lysozyme (10 ug/ml in 10 mM Tris pH 8)
   40 mls of solution II
   20 mls of ice cold PES

7. The bottles were shaken and stored on ice for 10 minutes.

8. Precipitated material was pelleted by centrifugation for 40 minutes at 4000 rpm at 4 ^{0}C. The run was stopped without braking.

9. The supernatant was separated by filtration through two layers of cheese cloth into a fresh centrifuge bottle.

10. Plasmid DNA and RNA were precipitated by addition of an equal volume of isopropanol (48 mls). Bottles were stored at room temperature for 1 hr.

11. Nucleic acids were pelleted by centrifugation at 5000 rpm for 25 minutes at room temperature.

12. After removal of the supernatant and removal of the last traces of liquid, pellets were resuspended in 3 mls of TE.

Purification of plasmid DNA by PEG precipitation

13. The 3 mls of nucleic acid in TE obtained previously was transferred to a 15 ml

Corex tube.

14. An equivalent amount of ice-cold 5 M LiCl solution was added to precipitate the RNA. The tubes were left to sit on ice for 1-2 hours.

15. RNA was pelleted by centrifugation at 10 000 at 4 ^{0}C for 10 minutes.

16. The supernatant was removed to a fresh 30 ml tube and an equivalent amount (6 ml) of isopropanol added.

17. The pellet obtained during centrifugation at room temperature and 10 000 rpm, was resuspended in 1 x TE containing pancreatic RNAase (20 µg/ml). The solution was transferred to an Eppendorf tube and stored at room temperature for 30 minutes.

18. Plasmid DNA was selectively precipitated by the addition of 500 µl 13 % (w/v) polyethylene glycol in 1.6 M NaCl. Tubes were left at 4 ^{0}C over night.

19. The following day plasmid DNA was pelleted by centrifugation for 5 minutes at 12 000 rpm in a microfuge.

20. Pellets were resuspended in 400 µl of TE and extracted once with phenol, once with phenol:chloroform (1:1), and once with chloroform.

21. The aqueous phase was transferred to a fresh microfuge tube and the following additions were made:

    133 µl of 7.5 M Ammonium acetate
    1 ml of ethanol (approximately 1 ml)

22. Plasmid DNA was recovered by centrifugation for 5 minutes at 12 000 rpm. Pellets were washed in 70 % ethanol.

23. Once the last traces of liquid had been removed/evaporated the pellets were resuspended in 500 µl of TE.