THE PHYSIOLOGY OF SKIN AND WOOL FOLLICLES OF FINEWOOL AND STRONGWOOL MERINOS.

by

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CHAPTER 2. FOLLICLE AND FIBRE CHARACTERISTICS OF STRONGWOOL AND FINEWOOL MERINOS

CHAPTER 3. SKIN BLOOD FLOW OF FINEWOOL AND STRONGWOOL MERINOS

CHAPTER 4. CUTANEOUS MICROVASCULATURE OF FINEWOOL AND STRONGWOOL MERINOS

ABSTRACT

Wool production differences between sheep maintained under similar environmental conditions appear to reside in the functioning of individual follicles. The investigations presented in this thesis utilise the differing wool producing abilities of two strains of Merino, finewool and strongwool Merinos. The relationships between wool production (on both a unit area and individual follicle basis) and skin and follicle characteristics, blood flow and microvasculature of the skin and incorporation of ^{3}H-glucose and ^{35}S-cystine by the skin were examined.

The differences in the structure and function of wool follicles and their association with fibre production were examined in 6 finewool Merinos (Camden Park) and 6 strongwool Merinos (East Bungaree). The strongwool Merinos produced 2.4 times more wool per unit area of skin and 3.5 times the volume of fibre per follicle than the finewool Merinos, when both groups were maintained under similar environmental conditions. The finewool Merinos had a higher follicle density, but a lower average volume of germinative tissue in the follicle bulb and the skin than the strongwool Merinos. The rate of cell production in the follicle bulb was greater in the strongwool Merinos than the finewool Merinos, but the proportion of bulb cells entering the fibre was not significantly different between strains. The number and volume of cells in the bulb and the cell length and volume of the cortical cells, and tended to be greater in the strongwool Merinos than the finewool Merinos, but also were not statistically different between strains due to a high 'between-sheep, within-strain' variation. Wool production per unit area of skin was highly correlated with the total volume of germinative tissue in the skin ($r = 0.91$; $P < 0.01$). This relationship was true for the strongwool and finewool Merinos and also in two groups of sheep from the same genetic base with one group selected using a WOOLPLAN index and the other a randomly-bred flock. It was concluded a) that genotype may determine the volume of potential mitotically-active follicle tissue in the skin, and b) that wool production on both a follicle and unit area of skin basis is not controlled by a single character, but rather is the result of a cumulative effect of a number of characteristics.

The physiology of the skin associated with high levels of wool production was further examined. In particular, blood flow through the skin of the strongwool and finewool Merinos was investigated using a laser Doppler velocimeter. This method was highly correlated with estimates obtained using ^{57}Co-microspheres ($r = 0.92$; $P < 0.01$) although the absolute values estimated by the microsphere technique were significantly greater ($P < 0.001$). Strongwool Merinos had a significantly greater rate of blood flowing through the skin than finewool Merinos ($P < 0.011$) and this was associated both with wool production per unit area of skin ($r = 0.58$; $P < 0.02$) and with follicle density ($r = -0.44$; $P < 0.1$). It was concluded that a) blood flow has an important role in the level of wool produced both within and between strains of Merino, and b) the laser Doppler velocimeter is a useful tool for the study of blood flow in the skin of sheep.

The microvasculature of the skin was examined using an infusion of silicone rubber into the deep circumflex iliac artery within the abdominal flank region of eight Merinos. The area of cascular tissue per unit volume of skin was independent of blood flow, wool growth and follicle density, both within and between strains of Merinos. The limitations of the technique used to examine the microvasculature, and its effect on the results are discussed.

The uptake of ^{3}H-glucose and ^{35}S-cystine by the skin and follicles was examined both *in vitro* and *in vivo* to determine if the follicles of the strongwool Merinos were capable of utilising the large nutrient pool supplied by the high rates of blood flowing through the skin. The skin and follicles of strongwool Merinos incorporate similar amounts of ^{3}H-glucose and ^{35}S-cystine per unit weight of skin than finewool Merinos. It was also found that the amount of radioactivity retained by the skin generally was not dependant on the amount of radioactivity supplied to the skin and follicles.

In summary, this study determined that strongwool Merinos have higher levels of wool production than finewool Merinos due to the presence of a large amount of tissue capable of producing fibre. This characteristic is maintained by a high rate of blood flowing through the skin. Wool production and blood flow through the skin are not influenced by the underlying anatomy of the microvasculature, nor is wool production restricted by the ability of the follicles to utilise nutrients from an extracellular pool for fibre production. Finally, the implications of this study and the usefulness in, and effect on, programs for selection of superior wool-producing genotypes are discussed.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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ABBREVIATIONS

ANOVA	Analysis of variance
AR	Analytical grade reagent
ATP	Adenosine triphosphate
BrdU	5'-bromo-2'-deoxyuridine
Ci	Curie
DMI	Dry matter intake
DNA	Deoxyribose nucleic acid
e.g.	For example
et al.	*et alia* (and others)
etc.	*et cetera* (and so on)
g	unit of gravitational field (acceleration of gravity)
g	gram
h	hour
IRS	Inner root sheath
kg	kilogram
LR	Laboratory grade reagent
M	Molar
mg	miligram
min.	minute
ml	millilitre
mM	millimolar
mmol	millimole
n	number of (samples)
ND	Not done
nm	nanomillimeter
nmol	nanomole
no.	number
nsd	no significant difference
^{o}C	Degrees Celcius
ORS	Outer root sheath

P	Probability
p.(pp)	page(s)
per se	as such
RO water	Reverse osmosis water
rpm	revolutions per minute
sem	standard error of the mean
SSA	Sulphosalacylic acid
TCA	Tricarboxylic acid cycle
TClA	Trichloroacetic acid
v/v	volume : volume
w/v	weight ; volume
µCi	microCurie
µg	microgram
µl	microlitre
µm	micrometre
µmol	micromole
y	year
%	percent
&	and
/	per
<	less than
>	greater than

CHAPTER 1.

INTRODUCTION

We stand upon the intellectual shoulders of the giants of bigone days

(Claude Bernard, 1855)

CHAPTER 1. INTRODUCTION

Objective measurement of various components of wool growth was introduced in the 1960's and is currently used to select superior wool-producing sheep for breeding purposes, with the aim of increasing overall wool production. These objective breeding programs have increased the amount of wool grown per sheep, but at a rate slower than that predicted by genetic studies (Rose, 1987). Rose (1987) attributed the lack of progress to the selection for wool production characters as being inefficient. The ability to identify superior genotypes would improve with the successful identification of key determinants of fibre production. This could provide an alternative to the current objective assessment of wool growth. The identification of characteristics of the skin associated with high levels of wool production would also allow selection of superior genotypes at a younger age. This may provide an additional cost saving, related to the early culling of unwanted animals and also increase the rate of genetic progress due to the mating of selected animals at a younger age.

Skin-based selection procedures have previously been unsuccessful due to a lack of knowledge of the complex interactions occurring in the wool follicle population (Davis & McGuirk, 1987). This suggests that

> "*...the conventional and somewhat simple-minded approach (to single selection of fleeceweight components) is inadequate in what we know as an interactive and dynamic set of physiological processes...*" (Davis & McGuirk, 1987).

There is, thus, a need to understand the physiology of wool growth and the underlying mechanisms that determine differences in efficiency of wool growth between sheep. This may then allow a more rapid improvement in wool growth genetics through the identification of new selection criteria.

Variations in wool production are evident between breeds, strains and individual sheep in the same flock. Differences in wool production between sheep in a flock may be due

to (i) the amount and quality of feed eaten,

(ii) the efficiency of digestion and absorption of nutrients from the alimentary canal,

(iii) the efficiency or pattern of general metabolism, or

(iv) the efficiency of wool follicle metabolism (Schinckel, 1960).

These variables would aptly apply to all sheep (i.e. sheep of different breeds and strains) in the same environment. Studies of sheep with different genetic potential for wool production have shown that the first three of the above biological variables are of minor importance in determining differences in wool growth, suggesting that the source of the differences reside in the functioning of the skin and its population of wool follicles. Evidence for this is presented in the following review of the literature.

The purpose of the present study was firstly to examine the relationship between follicle characteristics and wool production and, secondly, to obtain a better understanding of the underlying mechanisms associated with these characteristics in sheep with differing levels of wool growth. An understanding of these factors will increase the likelihood of identifying potential areas for manipulation and selection of superior wool-producing genotypes. The following review of the literature provides a brief introduction to the degree of variation of wool production between and within sheep populations, followed by a detailed description of the physiological factors which affect wool production on both a per unit area of skin and per individual follicle basis. Only relevant material published prior to the commencement of the present work (June, 1990) is considered in this review. More recent studies are examined in the following chapters. Special emphasis has been placed on the factors which cause differences in wool production **between** sheep, rather than treatments imposed on sheep at non-physiological levels or conditions known to alter wool production rates on **individual** sheep. Nevertheless, there are some instances where it has been necessary to examine characteristics which vary within sheep in order to understand the factors which may control wool production.

1.1. SHEEP USED FOR THE STUDY OF WOOL GROWTH

A large amount of variation is evident in the level of wool production between breeds, strains and individual sheep due to differences in both the rate of growth of the wool and the characteristics of the fibres. Studies have been conducted to determine the gross differences in

wool growth between breeds, with differences of up to 300% being observed between breeds. Such differences are the basis for a breeds' use in the sheep industry (Table 1.1.b). For example, the Lincoln, a carpet-wool sheep, produces twice as much wool as a finewool Merino (Daly & Carter, 1955), whereas the Border Leicester, a meat sheep, produces a third as much wool as the strongwool Merino (Langlands & Hamilton, 1969). These differences in wool growth are irrespective of feed regimen (Weston, 1959; Table 1.1.a,b).

Table 1.1.a. Wool production levels for different Merino strains with reference to the feeding conditions (either controlled intake trials with the individual animals fed in pens [pen fed] or the animals maintained outdoors with the feed intake being unknown [grazing])

Strain	Clean Fleece Weight (kg/y)	Reference (Feeding Regime)
Strongwool Merino	4.1 - 5.1	Dunlop *et al.*, (1966)
Mediumwool Merino	3.7 - 4.6	(pen fed)
Finewool Merino	3.0 - 3.6	
Strongwool Merino	3.7	Weston (1959)
Finewool Merino	2.4	(pen fed)
Strongwool Merino	3.7	Weston (1959)
Finewool Merino	2.3	(grazing)

The Australian Merino is distinguished from other breeds by a high ratio of secondary to primary wool follicles (S/P; primary and secondary follicles are described in detail in Section 1.3, p17). A high S/P ratio is associated with a high density of fibres per unit area of skin, which is in turn related to the low average fibre diameter typical of the Australian Merino (Table 1.2; Turner *et al.*,1986). Despite these distinguishing features of the breed, there is considerable genetic variation in the rate of wool production between strains of the Merino (Tables 1.1.a and 1.2). The development of the Merino strains arose from cross breeding as well as selection for a particular type of sheep for a certain environment, thereby resulting in differences in bodyweight, fleece production and wool qualities such as fibre diameter and other skin characteristics (Table 1.2) (reviewed by Hogan *et al.*, 1979).

Table 1.1.b. Wool production levels for different breeds with reference to the feeding conditions (either controlled intake trials with the individual animals fed in pens [pen fed] or the animals maintained outdoors with the feed intake being unknown [grazing])

Breed	Clean Fleece Weight (kg/y)	Reference (Feeding Regime)
Lincoln	5.9	
Corriedale	5.2	Daly and Carter (1955)
Polwarth	3.9	(pen fed)
Finewool Merino	3.0	
Border Leicester	2.7	
Dorset Horn	2.1	
Southdown	1.1	Langlands and Hamilton (1969)
Strongwool Merino	3.8	(grazing)
Finewool Merino	2.9	
Finewool Merino	2.4	Mullaney *et al.*, (1969)
Corriedale	3.1	(grazing)
Polwarth	2.9	
Coopworth	3.7	
Perendale	3.3	Joyce *et al.*, (1976)
Hi-fertility Romney	3.0	(grazing)
Romney	3.5	
Corriedale	4.3	
Coopworth	4.4	Sumner (1979)
Perendale	3.1	(pen fed)
Romney	4.5	
Border Leicester x Merino.	3.0	
Corriedale	3.5	
Polwarth	2.7	Atkins (1980)
Strongwool Merino	3.5	(grazing)
Peppin Merino	2.8	

Table 1.2. Wool characteristics of the six major Australian Merino strains (from Hogan *et al.*, 1979). Note the positive relationship between the upper values of follicle density and S/P ratio and the negative relationship between follicle density and fibre diameter

Strain	Bodyweigh t (kg)	Greasy fleece weight (kg/y)	Mean fibre diameter (μm)	Follicle density (no./mm^2)	S/P ratio
Saxon Merino	35-40	3.2	18-21	49-87	15-25
Spanish Merino	45-50	5.0-5.5	20-27	56	23
Peppin Merino	55	5.9-6.4	21-25	57-80	19-27
F.W. S.A.Merino[1]	60-65	6.4-6.8	21-25	42	
M.W. S.A.Merino[2]	60-65	6.4-6.8	21-27	54-65	16-19
S.W. S.A.Merino[3]	60-65	6.4-6.8	24-30	33	

[1] Finewool South Australian Merino

[2] Mediumwool South Australian Merino

[3] Strongwool South Australian Merino

Even within strains of Merinos there is a high degree of genetic variation (reviewed by Turner, 1977). In particular, flocks of sheep have been selected on single characteristics to develop 'genetically-different' sheep for experimental purposes. These flocks were established in the 1950's by the New South Wales Department of Agriculture at Trangie, by the C.S.I.R.O. at Cunnamulla in South West Queensland (Turner, 1958), which were later moved to Armidale (N.S.W.) in 1967, and at Roseworthy (S.A.). The initial flocks were selected for and against high fleeceweight (designated Fleece-plus and Fleece-minus lines, respectively), and flocks for other characteristics have since been established. For example, there has been selection for and against skin-folds (Dun, 1964), fibre number, staple length, fibre diameter, wool weight per unit area of skin (Rendel & Nay, 1978), primary follicle density, S/P ratio, and follicle depth (Jackson & Nay, unpublished; cited by Davis & McGuirk, 1987) The objectives of the flocks a) were to determine the heritabilities of clean fleece weight and its components and b) to establish flocks which exhibited large differences in the various selection traits.

1.2. PHYSIOLOGICAL FACTORS AFFECTING WOOL PRODUCTION PER UNIT AREA OF SKIN

Wool growth per unit area of skin responds to a number of factors such as nutrition, metabolism, internal and external environments* and genotype of the sheep. Whether a **differential response between sheep to these factors is causing differences in wool production** is unknown. The importance of each of these factors in inducing differences in wool growth due to a differential response by the sheep when subjected to the same conditions will be discussed below.

1.2.1. Internal and external environment

Wool production per unit area of skin varies between sheep of different ages due to change in nutrient demand from body growth during development and maturation, to wool production in the adult (Corbett, 1979). The age of peak wool production differs between breeds, for example Rambouillets, Romnelets and Canadian Corriedales reach maximum fleece weight at two years of age with production declining from four years of age (Slen & Banky, 1958). Most Merinos obtain peak wool production at three to four years of age (Brown *et al.*, 1966; Turner *et al.*, 1968; Mullaney *et al.*, 1969; Rose, 1974). It is therefore unlikely that the change in nutrient demand with stage of development would differ sufficiently between sheep of similar ages to be a major cause of differences in wool growth.

Pregnant and lactating ewes generally produce less wool per unit area of skin than non-pregnant sheep. This is due to an increase in the rates of protein synthesis and energy usage rates in the maternal animal (Blaxter, 1964) which results in a decrease in total wool production in the ewe. However, there is disagreement as to whether pregnancy or lactation has the greater effect on wool production (pregnancy greater - Brown *et al.*, 1966; Turner *et al.*, 1968; Mullaney *et al.*, 1969; Rose, 1974: lactation greater - Kennedy & Kennedy, 1968; Armstrong & O'Rourke, 1976) and this uncertainty may be due to confounding factors such as environmental differences between flocks. The effect of pregnancy and lactation on sheep with different wool growing capacities is still equivocal. For example, no difference was

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* Internal environment refers to factors such as physiological state, disease status and age, whereas external environment refers to factors such as temperature and photoperiod.

found in the effect of pregnancy nor lactation on wool production between a flock bred for high wool growth and a randomly bred flock (Brown *et al.*, 1966), whereas Williams (1979) found a greater depression of wool growth in pregnant Fleece-plus ewes than in Fleece-minus ewes.

The disease status of the sheep and the presence of parasites is known to affect wool production. It is unknown whether there is a differential response of genotypes varying in wool production to disease, i.e. are high-wool producers less susceptible to disease than low-wool producers ? The heritability of resistance to a number of diseases is moderate to high (reviewed by Donald, 1979; Nicholas, 1987; Piper, 1987; Raadsma & Rogan, 1987), and, *a priori*, there may be some difference in the level of immunity and/or susceptibility to infection, although this difference is unlikely to be a major cause of differences in wool production between genetically-different sheep.

Similarly, effects of temperature on wool production have not been investigated between genotypes differing in wool production. The effects of photoperiod on sheep in general have been reviewed extensively (Hutchinson & Wodzicka-Tomaszewska, 1961; Hutchinson, 1965; Ryder & Stephenson, 1968; Hutchinson, 1976) and it is known that Merinos are minimally affected by photoperiod in contrast to other breeds (Hutchinson, 1965). Ahmed *et al.* (1963) reported that the wool production of Fleece-plus and Fleece-minus sheep responded in a similar manner to photoperiod changes over a 12 month period. This suggests that changes in daylength are unlikely to be a major cause of difference in the amount of wool produced per sheep within the Merino breed.

1.2.2. Nutrition

There is uncertainty about the inter-relationship of bodyweight, feed intake, wool production and the efficiency of wool production*. Wool production is related to feed intake (Allden, 1979) and bodyweight (Ferguson, 1972), but these factors only account for a small proportion of the genetic variation in wool production between sheep. For instance, when liveweight differences are taken into account, there was no difference in intake between breeds (Daly & Carter, 1955), strains (Weston, 1959), or selection flocks (Ahmed *et al.*,

* Efficiency of wool production refers to the amount of wool produced per unit of dry matter intake.

1963; Williams & Miller, 1965; Dolling & Piper, 1968). This suggests that intake is determined by liveweight rather than intake *per se* determining the level of wool production. Williams (1979) concluded that differences in wool production between sheep within a flock may be due to intake differences between the sheep but it does not contribute to genetic variation. It is concluded that breeding for increased fleece weight has not resulted in any major changes in the energy-requirements needed to cause or maintain changes in liveweight.

There is general agreement that sheep with high rates of wool production tend to be more efficient convertors of feed to wool when comparisons between breeds (Langlands & Hamilton, 1969; Joyce *et al.*, 1976; Sumner, 1979), strains and selection flocks (Weston, 1959; Dunlop *et al.*, 1960; Ahmed *et al.*, 1963; Dunlop *et al.*, 1966; Williams & Winston, 1965; Williams, 1966; Dolling & Piper, 1968; Saville & Robards, 1972) and sheep within flocks (Schinckel, 1960; Dolling & Moore, 1960, 1961; Wodzicka-Tomaszewska, 1966) are conducted (Table 1.3). These results indicate that efficiency of feed conversion to wool contributes 50% to 90% of the variation in wool production. Williams (1987) concluded that the genetic capacity for wool production is not dependant on the quantity nutrients available to the sheep, rather it is the manner in which the nutrients are utilised.

The large differences in efficiency of wool growth may be a result of differing priorities on nutrient partitioning between wool production and bodyweight gain. For example, Border Leicester and Dorset Horn are large sheep breeds noted more for mothering ability and meat production than for wool production, and this may explain why they are relatively poor converters of feed to wool (Table 1.3) (Langlands & Hamilton, 1969). Also, breeds producing lambs with high weaning weights tended to be less efficient in converting feed to wool production when Perendale, Coopworth and Romney (high fertility and control flocks) sheep were studied (Table 1.3) (Joyce *et al.*, 1976). Wool growth rate is dependant on both the relative availability of nutrients to **all** tissues of the body and the relative rates of biochemical reactions competing for the same nutrients (Black & Reis, 1979). Black and Reis (1979) reviewed nutrient partitioning between wool growth and other body functions and applied available data to a simple computer model designed to predict wool production. They concluded that the maximum velocities and/or affinities for nutrients in tissues other than wool are likely to affect wool growth rate by affecting the nutrients available to the

follicle. This may generate differences in wool growth between sheep which have either different partitioning of nutrients or differences in the kinetics between tissues. Unfortunately, these partitioning theories have not been examined in sheep with different wool growing capacities.

Table 1.3. Wool growth rates, relative efficiency of wool production as a proportion of dry matter intake (DMI) and the feeding regimen (pen fed *vs* grazing) under which the experiment was conducted with different breeds and strains of sheep

Breed	Mean wool growth rate (g/d)	Relative efficiency. (Wool Growth/DMI) (% of finewool Merino)	Pen fed or grazing and reference
Lincoln	16.1	111-123	
Corriedale	14.3	102-109	Pen fed
Polwarth	10.7	106-110	(Daly and Carter, 1955)
Finewool Merino	8.2	100	
Border Leicester	7.3	73	
Dorset Horn	5.8	55	Grazing
Southdown	3.0	47	(Langlands & Hamilton,
Strongwool Merino	10.4	154	1969)
Finewool Merino	7.9	100	
Corriedale	11.8	120	
Coopworth	12.0	112	Pen fed
Perendale	8.5	86	(Sumner, 1979)
Romney	10.1	100	
Strain			
Mediumwool Merino	10.0-12.6	115-124	Pen fed
Strongwool Merino	11.3-13.9	124	(Dunlop *et al.*, 1966)
Finewool Merino	8.2-9.8	100	
Strongwool Merino	10.0	118	Pen fed
Finewool Merino	6.6	100	(Weston, 1959)
Strongwool Merino	10.0	119	Grazing
Finewool Merino	7.2	100	(Weston, 1959)

1.2.3. Metabolism

Differences in digestive function have been proposed as a possible mechanism for differing wool production level amongst sheep and may explain differences in efficiency of conversion of feed to wool (Schinckel, 1960). There are no differences in the digestive abilities of sheep genetically-different in wool production, nor are there any differences between sheep within a flock, when sheep are offered feed of varied digestibilities and energy and nutrient levels (Weston, 1959; Dunlop *et al.*, 1966; Piper & Dolling, 1969a; McClelland *et al.*, 1986). Comparisons between finewool and strongwool Merinos (Weston, 1959), and both Merino (Piper & Dolling, 1969b) and Romney (McClelland *et al.*, 1986) Fleece-plus and control flocks indicate that there is no difference in the digestive ability between the selection flocks. They conclude that the enhanced efficiency observed in high-wool producers are predominantly due to differences in the utilisation of absorbed nutrients after digestion for wool production, rather than due to differences in digestive ability.

Concentrations of blood metabolites, biochemical markers and genetic polymorphisms (i.e. distinct forms of a particular character which varies within a population, e.g. blood groups) and their relationship with wool production have been examined extensively (Table 1.4). Only those factors which are associated with wool growth, or those where there are conflicting results, will be discussed here. In most cases, the physiological effect of the metabolites is obscure. It is unlikely that differing levels of utilisation and/or absorption of the metabolites are factors determining differences in wool production between sheep. If the genes for the metabolites, markers and polymorphisms are closely related to genes determining the level of wool production, they may be useful as genetic markers.

Hough *et al.* (1988) found a higher level of plasma α-amino nitrogen in the Fleece-plus sheep than in the Fleece-minus sheep, but it is not known which amino acid contributed to the higher levels, nor was this difference consistently observed (Williams, 1987). The sulphur amino acids, methionine and cystine*, are the principle amino acids which stimulate

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* Cystine refers to both 1M cystine and/or 2M cysteine (or half-cystine) in this thesis unless the specific amino acid is intended, for example in reference to cysteine hydrochloride.

Table 1.4. Blood metabolites, biochemical markers and genetic polymorphisms which have been studied in association with wool growth

		Reference
Metabolites	Plasma cystine	Williams *et al.*, 1972a; 1986; Williams, 1976; 1984; Hough *et al.*, 1988
	Plasma urea	Sumner, 1979; McCutcheon *et al.*, 1987; Hough *et al.*, 1988; Cottle, 1988; Clark *et al.*, 1988
	Plasma creatinine	McCutcheon *et al.*, 1987; Cottle, 1988; Clark *et al.*, 1988
	Plasma glucose	Hough *et al.*, 1988; Cottle, 1988
	Blood acetate	Hough *et al.*, 1988
	Blood lactate	Hough *et al.*, 1988
	Plasma long chain fatty acids	Hough *et al.*, 1988
	Plasma hydroxybutyrate	Hough *et al.*, 1988; Cottle, 1988
	Plasma α-amino acid nitrogen	Hough *et al.*, 1988
	Blood protein	Cottle, 1988
	Plasma urea	McCutcheon *et al.*, 1987; Hough *et al.*, 1988; Clark *et al.*, 1988
	Blood cholesterol	Cottle, 1988
Markers	Erythrocyte glutathione	Hopkins *et al.*, 1975; Clark *et al.*, 1989
	Glutathione peroxidase	Cottle, 1988
	Alanine aminotransferase	Novikova, 1978; Perchikhin, 1980; Clark *et al.*, 1989
	Aspartate aminotransferase	Novikova, 1978; Perchikhin, 1980; Clark *et al.*, 1989
	Calcium	Cottle, 1988
	Phosphorous	Cottle, 1988
	Magnesium	Cottle, 1988
	Copper	Cottle, 1988
	Zinc	Cottle, 1988
	Potassium	Hopkins *et al.*, 1975
Polymorphs	Haemoglobin	Mayo *et al.*, 1970; Cottle, 1988
	R blood group	Mayo *et al.*, 1970
	Transferrin	Mayo *et al.*, 1970

wool growth (Reis, 1970; reviewed by Reis, 1989) and the metabolism and utilisation of these two amino acids have been studied extensively in the Merino. Levels of plasma cystine are consistently lower in Fleece-plus than in the Fleece-minus sheep (Williams *et al.*, 1972a; 1986; Williams, 1976; 1984; Hough *et al.*, 1988), despite the fact that the quantity of cystine entering/leaving the extracellular compartment does not differ between the two flocks (Williams *et al.*, 1972a; Williams, 1976). The reason for the different plasma cystine levels in sheep of differing wool growing capacity is still undetermined but it has been suggested that the incorporation rates of cystine into wool are unlikely to be responsible for differences in cystine concentration in the plasma (Williams, 1987).

A higher level of plasma urea is found in Border Leicesters than in finewool Merinos and this variation is attributed to differences in intake per unit metabolic bodyweight (Sumner, 1979). Conversely, there are significantly lower levels of plasma urea and creatinine in Romneys selected for greasy fleece weight compared to a control flock (McCutcheon *et al.*, 1987; Clark *et al.*, 1989; Thomson *et al.*, 1987). This is consistent with findings of low circulating urea concentrations in cattle of genetically-superior merit for milk/milk fat production (Tilakaratne *et al.*, 1980; Sejrsen *et al.*, 1984; Sinnett-Smith *et al.*, 1987) and sheep and pigs of superior merit for lean tissue growth (Mersmann *et al.*, 1984; Bremmers *et al.*, 1988; Carter *et al.*, 1989; Van Maanen *et al.*, 1989). In fact, canonical discriminant function of urea and creatinine, along with copper and magnesium, correctly classified 90% of Corriedale rams on the basis of greasy fleece weight (Cottle, 1988). These studies suggest a common genetic association between protein deposition and plasma urea, however, other studies have not found a similar relationship (Mackenzie *et al.*, 1988; Hough *et al.*, 1988). For example, Hough *et al.* (1988) found no difference between Merino Fleece-plus and Fleece-minus in levels of plasma urea.

Fleece-plus sheep had significantly higher levels of blood acetate and lactate than Fleece-minus sheep (Hough *et al.*, 1988). Williams (1987) suggested that the different concentrations of acetate were unexpected due to two factors. Firstly, the level of acetate in the blood is largely determined by acetate production during microbial digestion in the rumen (Pethick *et al.*, 1981) and, secondly, there is apparently only a small variation in microbial digestive capability between genotypes (Piper & Dolling, 1969b). The difference in acetate

concentration may result from metabolic differences between genotypes due to a difference in utilisation and/or partitioning of the acetate between the skin and other organs (discussed in Section 1.2.2).

Merino sheep with high rates of wool production tend to have high concentrations of potassium and low concentrations of reduced glutathione in erythrocytes (Hopkins *et al.*, 1975), whereas Romney rams selected for greasy fleeceweight had similar concentrations of reduced glutathione to those of control rams (Clark *et al.*, 1989). A genetic association has also been demonstrated between fleece weight and activities of the enzymes alanine aminotransferase and aspartate aminotransferase in plasma of Lincoln ewes (Novikova, 1978; Perchikhin, 1980). Selection for activities of these enzymes in plasma was considered to be a more effective means of increasing fleece weight than direct selection. However, Clark *et al.* (1989) found no significant difference in the level of enzyme activity between Fleece-plus and control lines of Romney rams, nor was there any correlation between enzyme activity and wool growth. The function or role of these metabolites in relation to wool growth are obscure, however, the metabolites may be indicative of differences in biochemical pathways within the Lincoln breed.

1.2.4. Endocrine status

A number of hormones is known to influence wool production and has been reviewed by Wallace (1979). The effect of hormones on wool growth was extensively demonstrated by Ferguson *et al.* (1965) who were the first to separate the effects of nutrition from those of hormones. However, there is little evidence that endocrine status differs significantly between genotypes and is a potential source of variation in wool production levels. This section only describes the hormones which have been examined in sheep with differing wool growing abilities and instances where the relationship between wool growth levels and the physiological endocrine status between sheep has been studied.

Cortisol is the major corticosteroid present in the plasma of sheep (Bassett & Hinks, 1969). There is no relationship between inherent levels of wool growth and cortisol levels, since the concentration of plasma cortisol measured in Fleece-plus and Fleece-minus sheep did not differ despite large differences in wool growth (Williams *et al.*, 1986). Hynd (1989b) supports this with unpublished information in which there was little effect of cortisol on the

uptake of ^{3}H-thymidine by the skin both *in vivo* and *in vitro*, and concluded that cortisol has little effect on wool growth at physiological levels (0.2 - 0.8μg/100ml). It is known that wool growth is stimulated by cortisol at concentrations slightly above physiological levels (< 2μg/100ml; Chapman & Bassett, 1970), although this may be an indirect effect of increased feed intake. Conversely, high doses of cortisol (> 3μg/100ml) decrease and often stop fibre growth (Lindner & Ferguson, 1956; Donald, 1979) due to a depression of mitotic activity and size of bulb cells (Chapman *et al.*, 1982).

Hough *et al.* (1988) measured levels of hormones in the the Fleece-plus and Fleece-minus ewes and found no difference in plasma growth hormone nor plasma insulin levels. They did observe a significantly higher level of plasma thyroxine in the low-wool producers than the high-wool producers. This is in agreement with others who compared Romneys selected for greasy fleece weight with a control flock of Romneys (Clark *et al.*, 1989). They found that the fleece-weight selected rams had lower plasma concentrations of thyroxine which were maintained, although not always at significant levels, throughout different nutritional regimes. More recently, Williams *et al.* (1990) compared thyroxine and tri-iodothyronine in wethers from the Fleece-plus and Fleece-minus flocks and found no difference between the flocks fed at two levels of nutrition. They concluded that genetic variation in the rate of wool growth was not due to differences in the activity of the thyroid gland. Others have found that only 15% to 30% of normal levels of thyroxine are needed to maintain wool growth at original rates in thyroidectomised sheep (Ferguson *et al.*, 1965; Maddocks *et al.*, 1985; Hynd, 1989b). Hynd (1989b) attributed the inability of thyroxine to stimulate wool growth above 'normal' levels to thyroxine having a facilitatory role rather than a regulatory role in wool production. This suggests that thyroxine may be like an 'on/off switch' rather than having an incremental effect. It is concluded that thyroxine is unlikely to be a factor causing differences in wool growth between sheep with different wool growth capacity.

Reklewska (1975) reported a positive correlation between wool growth rates and plasma catecholamine levels in ram lambs but not in ewe lambs. Adrenaline does not appear to affect cell division rate of the follicle bulb (Hynd, 1989b) and is unlikely to directly affect wool production, since adrenalectomy did not alter wool growth in sheep (Ferguson *et al.*,

1965). Others have indicated that intradermal injections of both adrenaline and noradrenaline produce a reduction in the uptake of ^{35}S-cystine (Cunningham *et al.*, 1979; unpublished data cited by Wallace, 1979). It is still equivocal whether changes in wool growth are due to a direct effect of the hormones or to the decrease in the blood supply resulting from vasoconstriction.

One of the major limitations of the hormonal studies of genetically-different sheep, is that only total plasma concentrations have been examined. Many hormones are released episodically and the frequency or height of the peak may cause differences in wool growth. Alternatively, hormonal response may be determined by the receptor activity or number. Thus the role of the endocrine status and its function in determining the level of wool production between genetically-different sheep can not be discounted.

1.2.5. Blood flow

High levels of wool production may be related to a greater apportioning of nutrients to the skin and its follicles or to the ability of some follicles to utilise available nutrients more effectively. Indirect evidence suggests that varied levels of blood flow to the skin may affect wool growth rates. For example, unilateral sympathectomy resulted in a greater rate of wool growth on the sympathectomised side which was attributed to the removal of the sympathetic action of causing vasoconstriction at low temperatures (Ferguson, 1949). Temperature changes which induce vasodilation or vasoconstriction, and thus alter the nutrient supply to the wool follicles, have also been reported to affect wool growth (Doney & Griffiths 1967; Lyne *et al.*, 1970; Jolly & Lyne, 1970). When skin temperature was used as an index of cutaneous circulation in a study of Romney Marsh sheep, it was concluded that differences in blood supply accounts for approximately 50% of the variation in wool growth over the body of the sheep (Cockrem, 1962). This is further supported by a computer simulated model which has shown theoretically that there would be a 50% increase in wool growth by doubling skin blood flow (Black & Reis, 1979).

Setchell and Waites (1965) examined the variation of skin blood flow in different regions of 7 Merinos and 4 Southdowns. They found that blood flow through the skin, expressed as a fraction of cardiac output, was not significantly different between the breeds. However, if the breed difference between each site are analysed separately, skin blood flow

in the back, midside, underbelly, lower shoulder and midrump differed significantly between breeds (Table 1.5). This suggests that there is generally a difference in blood flow to the skin in the major fleece-bearing areas, whereas in the regions where less wool is produced, i.e. the head, ears, and legs, there is no difference in the blood flow estimates.

Table 1.5. Blood flow through skin of Merino and Southdown sheep. (adapted from Setchell & Waites,1965)

Site	Blood flow (ml/min/g tissue ± s.e.m.)		t_{obs}
	Merino (n=7)†	Southdown (n=4)†	($t_{0.95,9}$=1.80)
Back	1.20 (0.07)	0.80 (0.16)	2.62*
Mid-side	1.13 (0.06)	0.77 (0.14)	2.88*
Belly	0.97 (0.04)	0.70 (0.08)	3.30*
Mid-shoulder	1.25 (0.07)	0.96 (0.13)	1.40
Lower-shoulder	1.19 (0.08)	0.92 (0.14)	1.88*
Mid-rump	1.20 (0.08)	0.71 (0.18)	2.90*
Lower-rump	1.03 (0.10)	0.99 (0.15)	0.24
Front leg	0.88 (0.05)	0.75 (0.13)	1.09
Rear leg	0.66 (0.05)	0.76 (0.10)	-0.40
Nose	1.02 (0.14)	1.27 (0.14)	0.28
Forhead	1.38 (0.08)	1.30 (0.15)	0.52
Ear	1.05 (0.14)	1.04 (0.03)	0.28

† n is the number of sheep in which blood flow through the skin was measured

* significant at $P < 0.05$

In studies of hair growth in the rat, it has been noted that blood vessel density is associated with hair growth and varies according to the stage of the hair cycle (Haddow and Rudall, 1945; Durward and Rudall, 1949). They found that there was a greater density of blood vessels at the advancing edge of the hair zone, implying a relationship between hair growth and blood flow.

Ryder (1956) observed anatomical differences in the arrangement of cutaneous capillaries between breeds of sheep. The Suffolk, Romney, Border Leicester and Masham have capillary networks within the dermis which are poorly developed, with many vertical vessels and few horizontal ones beneath the sub-epidermal net. However, the Welsh Mountain breed has a well-developed network, often with extra layers of horizontal vessels. Ryder proposed that these differences may reflect adaptation to certain environmental

conditions, and that a well-developed network is a primitive feature. This is contrary to the conclusion of Nay (1966) who suggested that the 'straight' follicles and the simple arrangement of the associated vessels may be considered a primitive feature. Despite this, it is evident that differences exist between the anatomy of the microvasculature of the skin and follicles, but whether this is related to genetically-determined levels of wool production is unknown.

1.2.6. Summary

This section examined the literature concerning the possible causes relating the functioning of the whole body to differences in wool production between 'genetically'-different sheep. It is concluded that metabolic differences such as digestion and absorption of nutrients from the gut, blood metabolites and endocrine status appear not to be associated with genetic wool-producing capacity. Thus, differences in wool production between sheep in similar environments possibly lie in the functional unit of wool growth, the follicle.

1.3. THE SKIN, ITS FOLLICLES AND FIBRE

Wool follicles are ingrowths of, and are continuous with, the epidermis (Fig. 1.1). At the base of each follicle there is a bulb enclosing the dermal papilla, the cells of which rarely divide. The cells present in the bulb divide rapidly and are pushed up the follicle shaft as the fibre is formed. The fibre is surrounded by three layers of cells collectively known as the inner root sheath (Fig. 1.1) which includes the inner root sheath cuticle, Huxley's layer and Henle's layer. Huxley's layer and the cuticle of the inner root sheath are unicellular in sheep, the cuticle lying closest to the fibre and interlocking with the fibre cuticle (Rogers, 1959; Chapman & Ward, 1979). Surrounding the inner root sheath is the outer root sheath which is continuous with the epidermis.

Follicles are described as being either primary or secondary (Wildman & Carter, 1939), determined by their order of initiation and distinguished histologically by their associated accessory structures. All follicles have sebaceous glands formed from the epithelial lining of the follicle and enclosed in a thin capsule of connective tissue. Eccrine

(sweat) glands, which are coiled, unbranched ducts opening onto the surface of the skin, and a branched muscle joining the follicle to the skin surface, known as the arrector pili, are associated only with primary follicles. Secondary follicles have neither eccrine glands nor an arrector pili muscle.

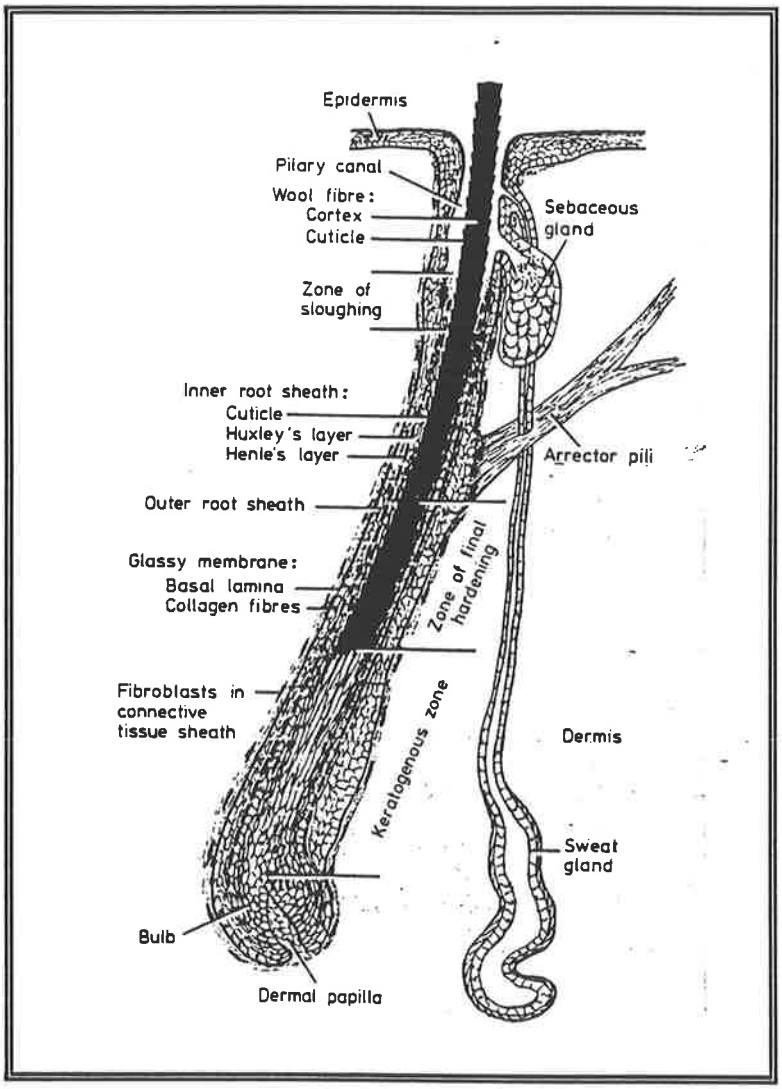

Figure 1.1. Diagrammatic representation of a primary wool follicle sectioned longitudinally (from Chapman & Ward, 1979). This figure demonstrates the anatomy of the wool follicle. The anatomy and processes which occur in many of the zones are further described in the text.

1.3.1. Follicle development.

The development of the wool follicle has been described in detail by Hardy and Lyne (1956a,b), Fraser and Short (1960) and Ryder and Stephenson (1968) and will only be covered briefly here. Pre-natal follicle initiation begins as a local aggregation of cells in the basal layer of the epidermis and an accumulation of underlying mesodermal cells (Hardy & Lyne, 1956a). An oblique plug forms from the proliferation of the epidermal cells which develops concurrently with the sebaceous and eccrine glands. The epidermal cells lengthen and flatten above the accumulated mesodermal cells and the base of the follicle invaginates enclosing the mesodermal cells, forming the dermal papilla. Elongated inner root sheath cells form a growth 'cone' above the dermal papilla and the 'cone' elongates and several streams of cells differentiate in concentric fashion. The outer layer hardens when its tip is about half-way up the follicle shaft to form the Henle's layer. The tip of the fibre begins to keratinise as it reaches the sebaceous gland, after which the growth 'cone' fragments. The tip of the fibre enters the fibre canal and then emerges from the skin surface.

All wool follicles are initiated before birth, but only the primary and some original secondary follicles produce emergent fibres *in utero*. Fraser (1954), Schinckel (1955) and Short (1955a) found that the development of both primary and secondary wool follicles was completed by the time lambs are several months old. It has been proposed that a reaction-diffusion system of epithelial origin controls hair follicle initiation and development (recently reviewed by Nagorcka & Mooney, 1989). Briefly, they propose a system whereby there are three, as yet, unidentified morphogens which react with each other and diffuse through the dermis. When the concentrations of the morphogens are either above or below a certain threshold, they act on the genome of differentiating cells and commit them to specific types. This may signal the development of the follicle (Nagorcka & Mooney, 1985; Nagorcka, 1986; Nagorcka *et al.*, 1987). Once a group of cells has been committed and differentiates into a particular cell type, the morphogens may alter the distribution of the compounds in the remaining tissue. This allows for the sequential development and positioning of the many structures of the follicle. The distribution of the components within both the dermis and follicle bulb determines the arrangement of primary and secondary follicles and their

subsequent growth (Nagorcka & Mooney, 1982; Nagorcka, 1984; Mooney & Nagorcka, 1985).

1.3.2. Follicle function and wool growth.

Wool follicles are dynamic organs in which proliferation, migration, protein biosynthesis, hardening and controlled degradation simultaneously in the fibre and inner root sheath (Chapman, 1986). Cell division occurs in both the region around the dermal papilla in the follicle bulb, and along the length of the follicle in the outer root sheath. The germinative region of the follicle is situated in the bulb of the follicle. Mitotic activity ends at a height of one cell layer above the apex of the papilla (Fraser, 1964), however there is some controversy as to whether all of this lower region is able to undergo division. Fraser (1964) and Adelson *et al.* (1990) observed bulbs exhibiting assymetric mitotic activity. The majority of reports are in agreement with Schinckel (1961), who found no concentration of mitotic activity in any particular segment of the bulb of follicles from sheep of 10 different phenotypes. It is generally assumed, and the contrary has not been observed, that the growth fraction of the bulb is unity, implying that all cells undergo division (Bullough & Laurence, 1958; Wilson & Short, 1979; Williams & Winston, 1987; Hynd, 1989a). Indeed, Fraser (1965) found no significant difference between the percentage of differentiating tissue in the bulb and the potentially mitotically-active tissue suggesting that all cells in the bulb are capable of division.

The destiny of the bulb cells is still a matter of some controversy, although it is generally accepted that the cells are committed to enter a particular part of the follicle or fibre at the time of their formation (Chapman & Ward, 1979; Chapman *et al.*, 1980; Potten, 1985; Hynd, 1989a). Differentiation of cells within the germinative region into outer root sheath cells, companion cells (Orwin, 1971), inner root sheath and fibre cuticle cells (Woods & Orwin, 1972) is detectable, suggesting that specific regions of the bulb do give rise to specific cell lines. The opposing view espoused by Montagna and Parakkal (1974), is that follicle cells are pluripotent and move randomly into one or other of the various layers of the follicle. However, there is little evidence to support this contention.

The outer root sheath cells proliferate along the length of the follicle. The cells of the outer root sheath have been identified as belonging to two populations (Chapman *et al.*, 1980). The proximal population forms the outer root sheath of the lower half of the follicle

and undergoes renewal in the region from the bulb to half-way up the follicle. This is the region where the greatest proliferation occurs in the outer root sheath. It is mainly in the suprabulbar region and around the keratogenous zone of the fibre where the outer root sheath is thickened (Chapman, 1971). The distal population comprises the outer layers of the outer root sheath above that previously mentioned, and the epithelium around the follicle lumen.

The inner root sheath cells move out of the bulb and up the follicle ahead of contemporaneously-formed cells of the hair and wool fibre in both humans and sheep (Epstein & Maibach, 1969; Chapman, 1971; Chapman *et al.*, 1980). The proximal population of the outer root sheath cells initially move towards the inner root sheath, then move distally within a peripheral population of the outer root sheath cells. After migration, the outer root sheath cells cornify, degrade and slough into the follicle lumen above the point where cells of the inner root sheath are shed (Chapman, 1971; Gemmel & Chapman 1971; Chapman *et al.*, 1980). The outer and inner root sheath cells that slough at any one time are not necessarily formed together due to the proliferation of the inner and outer root sheath cells at different levels in the follicle.

1.3.3. The fibre

The wool fibre consists of an outer cuticular layer of cells which surround the inner cortical region. The cortex is comprised of spindle shaped cells, arranged side by side with their long axis in the direction of the fibre. Intercellular cement and, often in fibres with a diameter greater than 40μm, a medulla complete the fibre architecture. The cuticle cells make up approximately 10% of the fibre weight and consist of three layers. Arranged from the outside into the cortex the layers are: (i) the epicuticle- a chemically resistant membrane complex (Lindberg *et al.*, 1949), (ii) the exocuticle and (iii) the endocuticle (Rogers, 1959). The cortical cells have finger-like ends which interdigitate with neighbouring cells (Rogers, 1959). The internal structure of the cortical cells is comprised of cell membranes, nuclear remnants and spindle shaped components of decreasing size ranging from macrofibrils, through microfibrils to protofibrils (Fig. 1.2.; Filshie & Rogers, 1961). These structures have been identified by light and electron microscopic examination of the intact fibre and the separated cells. Further analyses of the chemical composition of the fibre cells have been

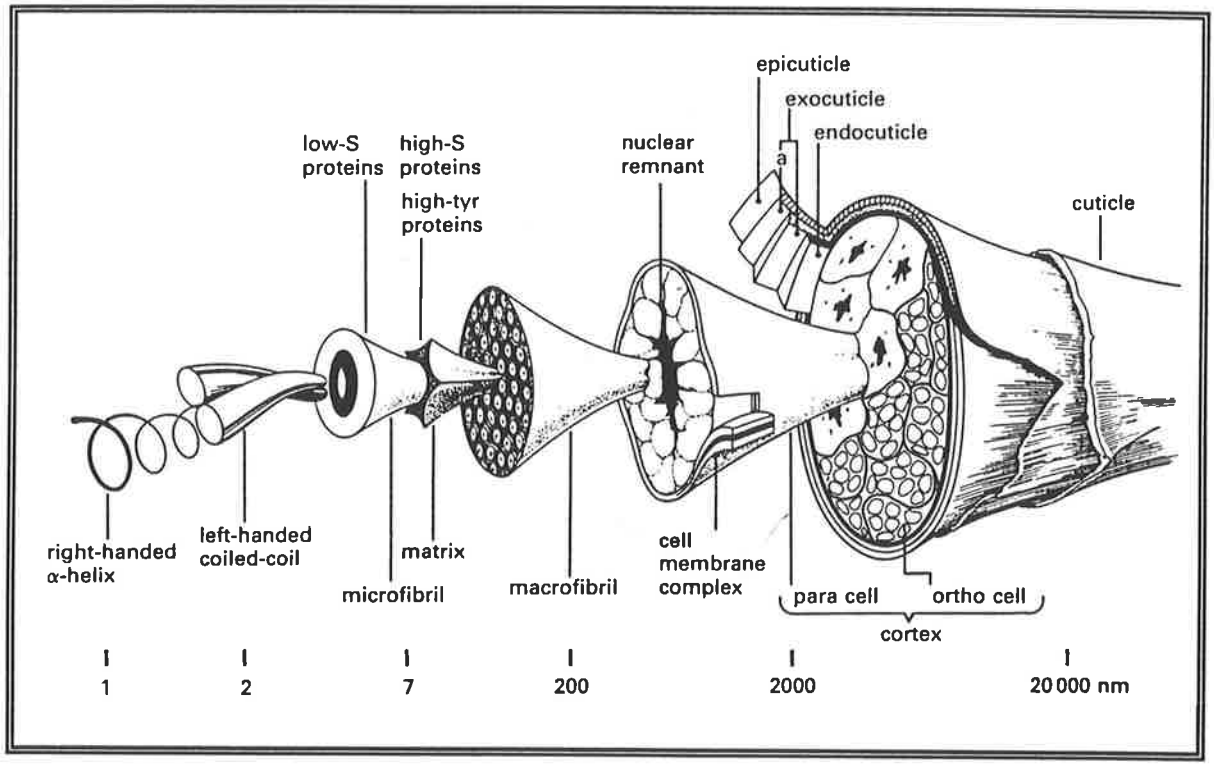

Figure 1.2. Schematic representation of the internal structure of a non-medullated wool fibre (from CSIRO Division of Protein Chemistry; adapted by Williams, 1991)

made of intact fibres (Ward *et al.*, 1955; Simmonds, 1956; Bradbury, 1960), separated cuticle (Elliot & Roberts, 1957; Bradbury *et al.*, 1966) and cortical cells (Ward *et al.*, 1955).

The microfibril - matrix complex within the cortical cells are an assembly of keratins which can be classed into several heterogenous groups; low sulphur, high sulphur and glycine-tyrosine proteins (Fraser *et al.*, 1972; 1976). The low sulphur proteins are the major component of the microfibril complex and contain all the methionine and most of the lysine present in wool (Marshall & Gillespie, 1977). The high sulphur proteins have a sulphur content greater than that of whole wool, but this varies considerably, depending on the nutritional status of the sheep. These proteins are rich in cystine, proline and serine and include the ultra-high sulphur proteins in which up to 30% of the amino residues are cystine.

Depending on the distribution of the macro- and microfibrils within the cells, cortical cells are classed as either ortho-, para,- meso- or metacortex with the first two being the most common. Orthocortical cells have a high microfibril:matrix ratio with approximately 60% of the cell being composed of microfibrils arranged in a whorl-like pattern within small discrete

macrofibrils. In contrast, the paracortical cells have a low microfibril density (approximately 30%) and the microfibrils are arranged in an open hexagonal array, while the matrix is heavily cross-linked with disulphide bonds (Rogers, 1959; Kaplin & Whitely, 1978; Orwin *et al.*, 1984; Powell & Rogers, 1986). Meso- and metacortex are of intermediary composition between ortho- and paracortex.

The complex arrangement of the cellular components and the quantities of amino acids needed for the keratinisation of the fibre provide much scope for causing differences in wool production between sheep. Indeed, fibres with large diameters tend to have a high proportion of large orthocortical cells in contrast to a high proportion of paracortex in fibres with low diameters (Orwin, 1989). *A priori*, fibre production is dependant on the supply, uptake and utilisation of nutrients by the follicle.

1.3.4. Nutrition of the follicle

Nutrient supply to the follicles for energy and protein synthesis is a consequence of both blood flow to the skin and the concentration of nutrients in the blood. Although blood flow to the skin has been studied extensively in relation to temperature control (e.g. Hales *et al.*, 1976; Bell *et al.*, 1983), little work has been undertaken relating blood flow to the supply of nutrients to the skin and wool follicles of sheep (see section 1.2.5).

The principles of follicle nutrition have generally been extrapolated from the effects of whole body nutrition on wool production. The major nutritional factors affecting wool growth are the amounts and proportions of amino acids, especially lysine, methionine and cystine, available for wool growth (reviewed by Reis, 1979; 1989). Lysine and methionine are essential amino acids obtained only from absorption from the gastrointestinal tract. Cystine is a non-essential amino acid for the sheep and can be obtained from transulphuration of methionine, primarily in the liver, and an extracellular pool in the skin (Downes *et al.*, 1976). Cystine does not necessarily have to pass through an intermediate stage in the skin since most is extracted directly from the circulatory system by the follicles (Downes, 1965).

The high rates of cell division and protein synthesis in the follicle do require high levels of energy, although the supply of energy to wool follicles is not an important factor limiting wool growth (Reis, 1969; Black *et al.*, 1973; Kempton, 1979; Reis *et al.*, 1988). Isolated wool follicles are capable of oxidising both actetate and glucose *in vitro* (Leng &

Stephenson, 1965). Acetate also accounts for half of the energy substrate utilised by the TCA cycle in isolated human hair follicles (Adachi & Uno, 1969). Other than these studies, there is little detailed information on energy metabolism in wool or hair follicles. Likewise there are no data concerning the uptake and absorption of nutrients and whether these factors differ between sheep.

1.4. SKIN CHARACTERISTICS ASSOCIATED WITH WOOL PRODUCTION

Clean wool growth can be described in terms of its components, which include smooth body surface, degree of wrinkle, mean fibre cross-sectional area, mean fibre length and mean number of fibres per unit area of skin (Turner, 1958). Genetic correlations between fleece weight and bodyweight indicate that the differences in clean fleece weight are predominantly associated with differences in wool production per unit area of skin rather than any large difference in surface area of skin (Brown & Turner, 1968; Turner *et al.*, 1970; Pattie & Barlow,1974). *A priori*, mean fibre cross-sectional area and fibre length determine the volumetric fibre production, whereas the mean number of fibres per unit area of skin is a function of the number of wool-producing follicles per unit area of skin (Williams, 1987). Genetic differences in wool growth are also related to differences in the morphology and function of the follicles in the skin of sheep. These will be discussed below.

1.4.1. Fibre and follicle number

The mean number of fibres per unit area of skin (fibre density) is a function of the number of active wool-producing follicles per unit area of skin. Follicle number, development and activity can be modified by prenatal, early postnatal and present nutrition (Schinckel, 1955; Short, 1955a,b: Weiner & Slee, 1965; Everitt, 1967). Maternal environment also has a strong influence on follicle initiation and development, although follicle number and activity are ultimately believed to be genetically-determined (Fraser & Short, 1960). For example, genetic differences were the largest source of variation for total follicle number in a study of the effect of a reciprocal transplantation of fertilised eggs from Welsh Mountain sheep and Lincolns (Weiner & Slee, 1965).

Fleece weight is correlated with fibre density in finewool, mediumwool and strongwool Merinos (Young & Chapman, 1958; Turner *et al.*, 1968; Nay & Hayman, 1969; Barlow, 1974; Brown & Turner, 1968) and accounts for up to 86% of the differences in clean fleece weight (Dun, 1958). Average fibre density differs more between individuals than between strains (Carter & Clarke, 1957a; Dunlop, 1962) but is breed related (Carter & Clarke, 1957a,b; Orwin, 1989) indicating that there is a large genetic component to the variability of fibre and follicle density. In Merino sheep, there is also a low, positive genetic correlation between wool weight and fibre number but this correlation decreases under selection systems due to the negative correlation between density and fibre volume (Brown & Turner, 1968).

1.4.2. Follicle distribution and morphology

In terms of follicle arrangement, an increase in wool weight per unit area is the result of an increase in the number of secondary follicles and a corresponding change in S/P ratios (Nay & Hayman, 1969; Barlow, 1974; Jackson *et al.*, 1975; Abouheif *et al.*, 1984; Williams & Winston, 1987; Miekle *et al.*, 1988) rather than a change in the number of primary follicles and follicle groups as was originally believed (Dun, 1958). In general, there is a greater variation in the number of secondary follicles than the number of primary follicles between sheep due to differences in the number of secondary derived follicles (Black, 1987).

Differences in the degree of curvature of follicles were first noticed and studied in Peppin Merinos (Nay, 1966; Nay & Johnson, 1967) and later in finewool Merinos (Nay & Hayman, 1969; Nay & Williams, 1969). They found that follicle chord length, curvature and depth are all correlated with wool production per unit area of skin. Experiments using the fleece weight selection flocks indicated that a genetic correlation exists between fleece weight and follicle curvature which is highly repeatable over a number of diets (Nay & Jackson, 1973; Williams & Winston, 1987) and ages (González *et al.*, 1983) but not affected by sex, year or litter size (Jackson *et al.*, 1975).

1.4.3. Selection for skin characteristics

Experimental flocks with a single skin characteristic as the selection criterion (Section 1.1) were initiated with the knowledge that certain characteristics were associated with wool

production. When selection is solely for clean fleece weight, there is an associated increase in the components of fleece weight (Turner *et al.*, 1970) (Table 1.6). However, in the case of selection for fleece weight with skin characteristics as the criterion, there was little increase in any line (Table 1.6).

Table 1.6. Percentage change in clean fleece weight and the components of clean fleece weight resulting from selection of Merino sheep with the components of clean fleece weight as the selection criteria

Selection character	% difference in component with fleece weight as criterion		% difference in fleece weight with component as criterion		Reference
	For	Against			
Clean fleece wt.	18.2	-14.6	-	-	Turner *et al.* (1970)
Wool weight¥	N.D§.		+13.1*	-13.5	Turner *et al.* (1970)
Fibre number¥	13.7	0.4	-0.4*	-7.3	Turner *et al.* (1970)
Fibre diameter	-1.0	-3.1	+1.1*	-5.4	Turner *et al.* (1970)
Staple length	7.0	-4.6	+5.4*	-16.1	Turner *et al.* (1970)
S/P ratio	N.D.		5.6†	N.D.	Rendel & Nay (1978)
Primary follicle no.	N.D.		10.0†	N.D.	Rendel & Nay (1978)
S/P ratio	8.4	N.D.	6.1†	N.D.	Heydenrych *et al.*, (1984)
Follicle depth	N.D.		-0.5*	N.D.	Jackson & Nay (unpubl.)#
Follicle number	N.D.		-1.7*	N.D.	Jackson & Nay (unpubl.)#
Tandem selection (depth/density)	N.D.		3.6*	N.D.	Jackson & Nay (unpubl.)#

¥ per unit area of skin
* difference from control as a percentage of control line
† percentage deviation of high line from low line
# cited by Davis & McGuirk (1987)
§ not done

The poor response of wool production change when fleece weight components were the breeding selection criteria may be a result of compensatory changes in other components of fleece weight (Rendel & Nay, 1978; Davis & McGuirk, 1987; Moore *et al.*, 1989). For example, selection for follicle number resulted in a decrease in fibre volume (Turner *et al.*, 1970). This response can be explained by the follicle competition theory (Fraser, 1951;

1953), which suggests that follicles compete with those around them for a fibre growth-limiting substrate. Therefore, if follicle density is high, competition for substrates also is high and the fibres produced will have a low volume. The density of the follicles will have a greater influence on the dimensions of the fibre rather than on the total amount of wool produced (Fraser & Short, 1952; Williams, 1987). Alternatively, but leading to the same result, it has been proposed that there is a genetically-determined development capacity which defines the amount of fibre-producing tissue (Moore *et al.*, 1989). They suggested that the effect of selection for a single skin characteristic was to change the distribution of fibre-producing tissue amongst initiation sites.

Sheep selected for high wool production would theoretically have stronger competition between follicles. This, then would lead to a larger negative relationship between fibre density and volume than those with lower wool production. In a situation where the limiting substrate is deficient, the increase in wool production would not be as great as when there are no nutrients limiting wool growth. This differential response to external stimulii is referred to as a genotype - environment interaction. If measurements are made under conditions where there is adequate feed, e.g. in pens or feed lots rather than under field conditions, the genotype - environment interaction may be compensated. The masking of genetic variation between animals within flocks by environmental variation has been observed in many cases (Hamilton & Langlands, 1969; Williams *et al.*, 1972b; Barlow, 1974) and may also partially explain why selection experiments have assymetrical responses in wool growth.

The conflicting results of Rendel and Nay (1978) and Heydenrych *et al.* (1984) (Table 1.6) may be related to the genotype - environment interaction. Rendel and Nay (1978) found no change in wool production when S/P ratio and number of primary follicles were the selection criteria and explained this result using Fraser's competition theory (Fraser, 1951; 1953). They stated that the increase in the follicle number per unit area of skin would not lead to increased wool production since the available nutrients are redivided amongst a larger number of follicles. However, this competition only takes place when there is a limited supply of substrates, either due to an internal or external inadequacy. Heydenrych *et al.* (1984) suggested that the competition theory was inoperative in their situation. They also

used the S/P ratio as their selection criteria and in their trial, the genotype - environment interaction was minimised and a predicted response recorded.

A hypothesis was proposed that follicle characteristics reflect some intermediate character in the chain of determination between the gene and the wool character. This led to the suggestion that it may be possible to predict wool production ability from skin structure (Jackson *et al.*, 1975). The characteristics were described in a causal model which indicated that the components of density, S/P ratio, depth and curvature accounted for 83% of the genotypic variation between sheep in wool production. By canonical regression, Jackson and his co-workers concluded that there were three independent pathways, i.e. three genes or groups of genes, of genetic control. First, via S/P ratio controlling fibre density; secondly, via straight deep follicles which control fibre length and; thirdly, via the number of primary follicles and the follicle group density which controls both diameter and density. On this basis, it was suggested that selection lines for some of these characters should be established to determine the practicality of using them as selections for fleece weight. However, in unpublished work from Jackson and Nay (cited by Davis & McGuirk, 1987) there was very little change in clean fleece weight when high follicle number and depth were individually selected, although when both were selected together (tandem selection) there was a slight increase in clean fleece weight (Table 1.6).

The majority of the selection flocks have been small and unreplicated leading to problems such as inbreeding and random genetic drift (Davis & McGuirk, 1987). Despite these limitations, it appears that the individual components of clean fleece weight are highly interrelated and the relationship of skin characteristics to wool production is unclear. Since single selection for any of these characteristics has not affected total wool production it is necessary to understand the **physiological** relationship between attributes of the follicle and wool growth.

1.5. INFLUENCE OF THE FOLLICLE ON WOOL PRODUCTION

The early work of Schinckel (1961; 1962) provided the foundation for the study of the relationship between wool production and follicle morphology and kinetics. Schinckel (1961) compared ten sheep with different fleece phenotypes and concluded that at least two thirds of the difference in the rate of fibre growth was due to differences in the number of

cortical cells produced in the bulb and the remainder due to differences in cortical cell volume. This was supported by a second experiment involving one sheep fed two levels of nutrition to create different rates of wool growth (Schinckel, 1962). He determined that a 156% increase in wool production was the result of a 56% increase in the rate of cell production and a 64% increase in the volume of individual fibre cells. He surmised that differences in the number of cortical cells produced were due to differences in the number of germinal cells rather than germinal cell volume or cell turnover rate, despite a small difference in turnover rate.

1.5.1. Follicle morphology

A priori, fibre production is determined by the rate of division of the bulb cells, the proportion of these cells entering the fibre *per se* and the size of cortical cells forming the fibre. These are, in turn, potentially determined by the volume and the number of cells in the bulb and the number and rate of cells undergoing division at any time. Due to the difficulty of quantifying these factors, there is a dearth of data describing all of the possible characteristics associated with wool production and, of the available work, there is little general agreement between authors as to the mechanisms responsible for different levels of wool production (Table 1.7). Many of the experiments have used nutrition to generate differences in wool production and these results will be discussed with reference to possible genotypic effects when appropriate.

Table 1.7. Changes (percentage) in the volume of fibre produced (fibre volume), the amount of germinative tissue in the bulb (germ tissue), the number of cells per bulb (no cells/bulb) and the mean volume of these cells (bulb cell vol) associated with improved nutrition

	Fibre volume*	Germ tissue*	No cells/bulb*	Bulb cell vol.*
Short *et al.*, (1965)	156	8†	12	21
Wilson & Short (1979)	109	46#	40	3
Williams & Winston (1987); Fl +	43	3†	N.D§	N.D
Williams & Winston (1987); Fl -	7	-4†	N.D	N.D
Hynd (1989a)	33	30#	20	N.D

* Percent change associated with an increase in nutrition

† Measured as diameter

Measured as volume

§ Not done

Generally, the rate of wool production is positively correlated with the amount of germinative tissue in the bulb, whether germinative tissue is described in terms of either bulb volume or diameter (Table 1.7 - Fraser, 1965; Henderson, 1965; Short *et al.*, 1965; Wilson & Short, 1979; Hynd, 1989a). For example, Wilson and Short (1979) found that bulb width and daily fibre production were significantly correlated ($r = 0.80$, $n = 10$). The absolute percentage change of germinative tissue present in the skin from low to high levels of nutrition varies between experiments (Table 1.7) and seems to be dependant on how germinative tissue is described. As an example, Williams and Winston (1987) measured bulb diameter when they compared Fleece-plus and Fleece-minus sheep fed at two levels of nutrition. There was an increase in fibre production associated with improved nutrition in the Fleece-plus sheep and a small, non-significant increase in the fibre production of the Fleece-minus sheep (Table 1.7). Despite these differences in wool production, there was no significant difference in follicle bulb diameters between the two dietary levels (Table 1.7), in agreement with Short *et al.* (1965) who also used bulb diameter to describe germinative volume. This is not surprising since the bulb approximates a sphere, thus a small difference between the diameters of two bulbs would be equivalent to a much larger volume change. This theory is supported by the conclusions of Henderson (1965) who dissected individual follicles taken from three groups of Romney sheep fed at three nutritional levels. After measuring the lengths and widths of the fibres, follicles and dermal papillae, he found that there was a significant positive correlation between fibre length and follicle dimensions, but there was no relationship between the latter and fibre diameter. He concluded that follicle dimensions (i.e. length and width) have only a small and inconsistent influence on fibre size between sheep. It is concluded that bulb diameter, as a single dimension measurement, does not vary as much as bulb volume, as a 3-dimensional measurement, when there is a change in wool production induced by nutrition. When all the results are considered, it appears that there is a significant relationship between wool production and the volume of germinative tissue in the bulb.

Whether the volume of germinative tissue in the bulb is determined by the number of cells, the volume of individual cells in the bulb or a combination of the two remains unclear (Table 1.7). For example, when a single Merino ewe was subjected to two levels of nutrition

(Short *et al.*, 1965), there was an increase in the volume of individual bulb cells (21%) with a smaller increase in bulb cell number (12%). In contrast, Fraser (1965) compared the germinative volume of two Romney sheep at two different times (July and October) during which an increase in wool production is presumed*. He measured the cross-sectional area of serial bulb sections and calculated germinative volume from this and the section thickness. Fraser concluded that the change in germinative volume was a result of a change in bulb cell number rather than cell volume, in agreement with results obtained by Wilson and Short (1979). The latter found a large difference in bulb volume (50%) and cell number (41%), and these two parameters were highly correlated ($r = 0.81$, $n = 10$), but there was no difference in the mean bulb cell volume between two groups of five Peppin-Merino sheep fed at a low and a high level of nutrition (Table 1.7). Although the total number of cells in the bulb was not correlated with daily fibre production, an analysis of variance indicated that cell number was influenced by feed intake (Wilson & Short,1979).

Hynd (1989a) observed similar changes to those of Wilson and Short (1979) when he examined five strongwool Merinos, a Corriedale and a finewool Merino at two levels of nutrition . He reported a 30% increase in the average volume of germinative tissue which was associated with a 33% increase in wool growth and an increase in the number of cells in the bulb. However, cell volume was assumed to remain constant at 426μm^3 (derived from Wilson & Short [1979]), and this was used to determine cell number. There is no evidence for constant cell size, so this assumption must be questioned.

1.5.2. Follicle kinetics

The response of fibre production to nutrient intake should reflect a change in the activity of the follicle, i.e., the number of cells produced by the follicle bulb. Fraser (1965) speculated from his work involving the dissection and measurement of individual follicles from Romney, Merino and Border Leicester sheep, that the one of the main causes of variability between sheep was a difference in the rate of cell division. He concluded that this would control the relationship between fibre length and diameter, since the follicle can readily

* There are many reports which suggests that there is an increase in wool production in Romney sheep over this period of time in New Zealand, e.g. Storey & Ross (1960), Sumner & Wickham (1969), Bigham *et al.* (1978).

accommodate kinetic changes without large changes in papilla and bulb dimensions. The kinetics of the follicle bulb are described in three different terms; (i) the number of cells produced per unit time, (ii) the turnover time of the bulb, and (iii) the mitotic density. Each of these are interrelated, however they are not associated with wool growth in a similar manner. The relationship of each with wool growth will be discussed separately below.

Most work has indicated that there is an increase, although somewhat varied, in the number of cells produced in the follicle bulb associated with an increase in wool growth due to improved nutrition (Table 1.8). There tends to be a linear association of fibre production with cell production rate when the latter is expressed as the number of cells produced per hour per bulb (Schinckel, 1962; Short *et al.*, 1965; Wilson & Short, 1979; Hynd *et al.*, 1986; Hynd, 1989a). Wilson and Short (1979) found that the rate of cell proliferation in the bulb was highly correlated with the volume of daily fibre production, in agreement with results of others. Hynd *et al.* (1986) generated a range of wool growth rates by switching one Merino from a high level of feed intake to a low level, a second Merino from a low level of nutrition to a medium level and feeding a third Merino on a high energy - high protein ration. They found a positive relationship between daily fibre production and the number of cells produced per bulb. Finally, the number of cells dividing per hour per bulb accounts for a significant proportion of the variance in wool production under multiple linear regression (Wilson & Short, 1979; Hynd, 1989b) which suggests that the number of cells dividing in the bulb plays an important role in determining fibre production.

Turnover time describes the time it takes the total population of cells in the bulb to renew themselves. It is a function of the number of cells in the bulb and the number of cells entering mitosis over time (Fraser, 1965), however the relationship of this with fibre production is unclear (Table 1.8). Some workers found a negative relationship between fibre production and turnover time, i.e. as fibre production increase, the cell population replaces itself more rapidly resulting in a shorter bulb turnover time (Short *et al.*, 1965; Fraser, 1965; Wilson & Short, 1979). However, turnover time did not account for any variance in wool production in a linear regression model (Wilson & Short, 1979), and others have found no correlation (Fraser, 1965; Hynd, 1989a). Fraser (1965), in his experiment involving two Romneys, reported one sheep which showed an increase in turnover time due to a change in

Table 1.8. Changes (percentage) in the volume of fibre produced (fibre volume), the number of cells undergoing division (Mitotic number) and the turnover time of the bulb associated with improved nutrition

	Fibre volume*	Mitotic number*	Turnover time*
Short *et al.*, (1965)	156	63†	- 31
Wilson & Short (1979)	109	81†	- 26
Williams & Winston (1987); Fl +	43	12#	N.D§
Williams & Winston (1987); Fl -	7	25#	N.D
Hynd (1989a)	33	35†	0

* Percent change associated with an increase in nutrition

† expressed per bulb per hour

expressed per unit volume of germinative tissue

§ not calculated

mitotic density, whereas the other animal showed no change in mitotic density and thus no change in turnover time. The latter may once again represent no change in wool production since no measurement was made of wool growth. Similarly, Hynd (1989a) assumed bulb cell volume was constant and used this assumption to determine bulb cell number and thus turnover time, which in turn may have affected his estimates of turnover time. The relationship between wool growth and turnover time is equivocal, partly due to the lack of studies. Turnover time, however, does have the potential to alter fibre production since it has been shown that turnover time varies between follicles within species and is influenced by nutrition. For example, the turnover time of human-hair follicles has been reported to range from 23 hours (Van Scott *et al.*, 1963) up to three days (Hoffman, 1953). Mather and Geo (1976) found that undernourished rats had poor hair growth (both in diameter and in length) compared to control rats and this was associated with an increase in cell turnover time due to an increase in the G_2- and S-phases of the cell cycle.

When the activity of the follicle is expressed as the number of cells undergoing division per unit volume of bulb tissue, i.e. mitotic density (Fraser, 1965), the relationship with wool production is less clear. It should be noted that mitotic density is not a function of turnover time unless the number of cells in the bulb is taken into account. Schinckel (1961) found a close association between the diameter of the follicle bulb and the number of cell undergoing mitoses$^{1/3}$ in sheep with different fleece phenotypes. From this, he concluded

that there was a constant mitotic density despite large differences in wool growth between genotypes. Fraser (1965) also found no relationship between mitotic density and the volume of germinative tissue (and thus wool growth) in a Merino, but there was a negative relationship between mitotic density and germinative volume in a Border Leicester and a Romney. He suggested that the negative relationship was due to an increase in the number of cells in the bulb. To add further confusion, Fraser (1965) also reported a marked increase in mitotic density in one Romney but no change in another when estimated at two different periods (as described earlier). However, it is difficult to make any clear conclusions concerning the relationship with wool growth in this experiment as growth was not actually measured. Thus it is possible to speculate that there was no change in wool growth in the sheep which exhibited no change in mitotic density. However, Williams & Winston (1987) did find a difference, although not significant, between mitotic density at different feeding levels associated with the increase in wool production (Table 1.8). It is concluded that the number of cells undergoing division per unit volume of germinative tissue may be related to wool growth but it does not appear to have an important role in the control of wool growth.

It can be surmised that an increase in fibre production in response to an increase in nutrient supply is due to two components - an increase in germinative volume and a concomitant increase in the number of bulb cells proliferating per unit time. Nevertheless, it is unclear whether this increase in mitotic activity is due to a change in the number of cells dividing at once or whether the cells already present are dividing at a faster rate, i.e. if there is a change in cell division rate, cell turnover time or both.

1.5.3. Distribution of bulb and fibre cells

Fibre production is apparently an inefficient process since less than half of the cells which are produced in the bulb enter the fibre cortex with the remainder being incorporated into the inner root sheath (Table 1.9; Short *et al.*, 1965; Wilson & Short, 1979; Hynd, 1989a). Short *et al.* (1965) reported a slight increase (14.6% up to 18.3%) in the proportion of bulb cells entering the fibre on an improved plane of nutrition when samples from one sheep on two planes of nutrition were compared. However, Wilson and Short (1979) did not find any relationship between feed intake, wool production and the proportion of cells entering the fibre. Hynd (1989a) found that the proportion of cells entering the fibre varied widely

between sheep on both a high and a low plane of nutrition, but was a repeatable character within-sheep at the two levels of nutrition. In fact, the mean values of cellular efficiency for the low and high planes of nutrition were 31% and 32%, respectively, suggesting very little nutritional influence. Both Wilson and Short (1979) and Hynd (1989a) concluded that the proportion of bulb cells contributing to fibre cortex was independent of nutritional level, thus suggesting a genetic control. This was further supported by Hynd and Scobie (unpublished, cited by Black, 1987) who found that up to 51% of bulb cells enter the fibre cortex in the Lincoln compared to only 23% in the Merino and 16%* in the Suffolk. These percentages are related to the type of wool produced by the individual breeds of sheep, i.e. the Lincoln is a long-wooled breed, the Merino a medium-length breed and the Suffolk a short-wooled breed. Similarly, from results presented by Williams and Winston (1987; recalculated by Hynd, 1989a), it can be determined that the ratio of cortical cells produced per hour to the number of cells undergoing mitosis per bulb was greater in the Fleece-plus sheep and this was not nutritionally dependant.

Butler and Wilkinson (1979) reported a correlation between the level of wool produced per unit of dry matter consumed and the proportional area of the fibre compared with the inner root sheath plus fibre (production ratio) in Corriedales. They suggested that this ratio may be used as a simple means of identifying efficient wool producers. However, inherent in this ratio are two assumptions, a) that the relative rates of migration and the change in size are the same for both the inner root sheath cells and the fibre cells, and b) that wool production per unit area of skin is dependant on cell distribution with no interaction with other characteristics. In Merino sheep, there was no correlation between production ratio and cell distribution (Hynd, 1989a). The production ratio did not account for a significant proportion of the variance in fibre production per follicle which suggests the assumptions of Butler and Wilkinson (1979) may be invalid. In fact, when skin samples injected intravenously with

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* This value is confounded by the presence of the medulla in the fibre. When the number of cells that enter the fibre per hour is calculated using ratio of the volume of cortical cells to the volume of fibre produced per hour the presence of the medulla is not taken into consideration. Thus, the proportion of bulb cells entering the fibre may be an underestimate.

Table 1.9. Changes (percentage) in the volume of fibre produced (fibre volume), the volume of individual cortical cells (cortical cell vol) and the proportion of bulbs cells which enter the fibre (cellular efficiency) associated with improved nutrition

	Fibre volume*	Cortical cell vol*	Cellular efficiency	
			Low	High
Short *et al.* (1965)	156	19	15†	18†
Wilson & Short (1979)	109	37	15†	13†
Williams & Winston (1987)§; Fl +	43	24	2.2#	2.1#
Williams & Winston (1987)§; Fl -	7	-16	1.5#	1.5#
Hynd (1989a)	33	5	31†	32†

* Percent change associated with an increase in nutrition

† Percentage number of mitotics per bulb per hour to number of cortical cells produced per hour

\# Ratio of cortical cells produced per hour to bulb cell mitotic density

§ As estimated by Hynd (1989a)

^{3}H-thymidine were examined and the movement of cells was studied by autoradiography (Chapman *et al.*, 1980) it was found that the inner root sheath cells migrate out of the bulb ahead of fibre cells formed contemporaneously. It has been suggested that this difference in migration rates would not be so great as to allow the production ratio to overestimate the various partitioning of cells to fibre and inner root sheath (Williams & Winston, 1987) and may provide a very general indication of bulb cell distribution.

The volume of individual cortical cells in the fibre is associated with nutrition (Table 1.9), but there tends to be more variation between individuals within nutritional levels, suggesting an internal environmental and/or genetic influence (Short *et al.*, 1965; Wilson & Short, 1979; Williams & Winston, 1987; Hynd, 1989a). For example, Wilson and Short (1979) found a high positive correlation of cortical cell volume with fibre growth, however they noted that cell length and diameter varied independently both within- and between-sheep. They concluded that cortical cell volume is not significant in determining variation in fibre production between sheep, which is further supported by the results of Hynd (1989a).

The determination of cortical cell volume and the distribution of cells between the fibre and the inner root sheath may be confounded by cortical cell composition changes. The determination of cortical cell type appears to be under genetic control and tends to be related to

fibre size (Orwin *et al.*, 1984; Black, 1987). The effects of nutrition on cortical cell volume and type are debatable (Wilson & Short, 1979; Williams & Winston, 1987; Hynd, 1989a,b). It is uncertain whether different degrees of ortho- or para-cortical segmentation may have a role in the change in cortical cell volume, thus adding to the nutrition x genotype interaction observed by Williams & Winston (1987). Hynd (1989a) reported an increase in the proportion of paracortex in the fibre when a high protein diet was offered. He noted that his results contrasted with the apparent tendency for large fibres to be composed predominantly of orthocortical cells in Romneys and Perendales (Ahmad & Lang, 1957; Orwin *et al.*, 1984). However, Hynd (1989a) suggested that the high level of sulphur-amino acids supplied in the diet enables the production of high sulphur proteins found in paracortical cells. He also observed an apparent genetic effect on the cortical composition in the finewool Merino which had high levels of paracortex at both levels of nutrition. Hynd (1989a) concluded that the ultimate size of the cortical cells do not always contribute to variations in wool growth, possibly because changes in nutrition may be reflected in cell type rather than cell size.

1.5.4. Genetic studies

There has only been one study which examines follicles and wool growth in sheep genetically-different in wool production (Williams & Winston, 1987). Williams and Winston (1987) compared two groups of sheep from the Fleece-plus and Fleece-minus flock and found that 20% more cells were present in the fibre cortex of the high-wool producers despite the average volume of cortical cells not differing between genotypes. There was a difference between flocks in the area of actively-dividing tissue in the skin but there was no difference between genotypes in the rate of cell production per unit volume of bulb tissue after an intradermal injection of colchicine or follicle bulb diameter. When they re-examined a group of slides sectioned at 4μm it was found that mitotic density was lower in the Fleece-plus sheep. This anomaly between the two samples as well as the unexpected results of no relationship of wool growth with cell division between the two flocks needs to be re-examined.

Authors often attribute certain characteristics to being under genetic control particularly when there is greater variation between sheep within a treatment than between treatments (Short *et al.*, 1965; Wilson & Short, 1979; Williams & Winston, 1987; Hynd, 1989a).

Follicle and fibre characteristics which have been suggested as being determined by genotype include the production of cells in the bulb, (Schinckel, 1961), bulb volume (Fraser, 1965; Short *et al.*, 1965; Wilson & Short, 1979), number of cells in the bulb (Wilson & Short, 1979), the proportion of bulb cells entering the fibre (Wilson & Short, 1979; Hynd, 1989a) and the volume of cortical cells (Williams & Winston, 1987; Hynd, 1989a).

A point to note in the experiment of Wilson and Short (1979) was that the experimental design consisted of nine sheep fed a mix of lucerne hay and oats at a rate of 10, 20 and 30g/kg bodyweight with 4 sheep each being in the low and medium levels and one in the highest level. The sheep on the highest level of nutrition has been grouped with the medium group in the present comparison and the tenth sheep, which was feed 15g/kg bodyweight of lucerne hay, was included in the analysis as being on the low ration. The interesting feature of the results was that the single sheep in the highest feed group had the highest rate of wool production but the volume of germinative tissue and number of cells in the bulb were less than the means of the medium feed group. These results suggest that genotype may be a factor determining the high rates of wool production.

1.5.5. Summary

Nutrition has been used extensively to generate differences in wool production and to observe associated changes in follicle and fibre characteristics. Genetic studies have been limited to speculation from nutritional studies and one comparison of the Fleece-plus - Fleece-minus lines. The evidence from the nutrition experiments suggest that wool growth changes are associated primarily with changes in rate processes (i.e. cell kinetics) since the follicle can accommodate these changes more easily than changes in follicle size. It should be noted, nonetheless, that dimensional changes are also observed. These presumptions are likely to be true with genotypic differences in wool growth, but the results are inconclusive.

1.6. CONCLUSION.

Differences in wool production between sheep can be attributed to environmental, nutritional, physiological or genotypic variation. However, if all non-genetic factors i.e. environment, nutrition and physiological state, are held constant wool growth still varies considerably. This difference is generally attributed to genotype. Many studies have been

conducted to determine the factors associated with high-wool producing genotypes. At present, there seems to be no particular metabolic or physiological character associated with high-wool production.

Differences in wool production are associated with certain skin characteristics such as follicle depth, curvature and density. However, these characteristics do not **cause** differences in wool production, as observed by single selection experiments for these characteristics, rather, they are merely **associated with** wool production. Other metabolic differences such as digestion and absorption of nutrients from the gut, blood metabolites and endocrine status, appear not to be associated with high-wool producing genotypes. Thus, differences in wool production between sheep in similar environments may lie in the functional unit of wool growth - the follicle.

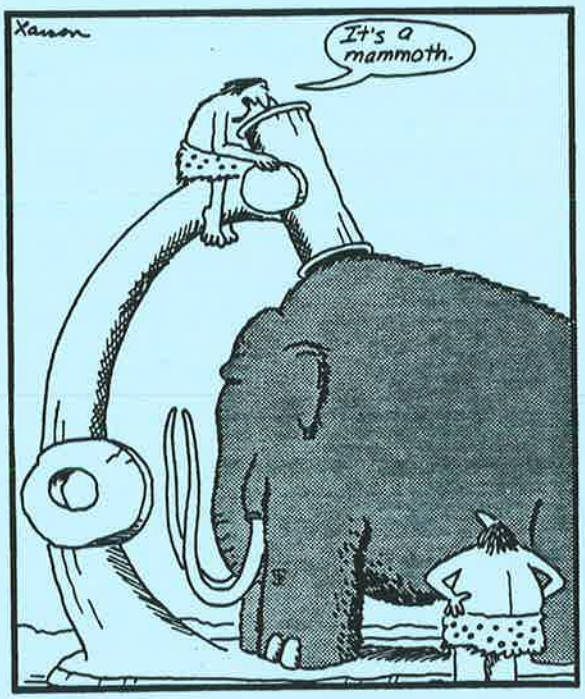
At present there is only one study and a body of indirect evidence attained from nutritional experiments, concerning the relationship of individual follicle characteristics with wool growth. The present study was undertaken to examine the physiological basis of differences in wool production at the level of the follicle, in sheep differing widely in wool growth rates in similar environments. Thus, major objectives of the present study were;

(i) to determine follicle characteristics associated with high versus low levels of wool production, and

(ii) to examine the supporting physiological characteristics which maintain high levels of wool production, in particular blood flow to the skin and nutrient uptake by the skin and follicles.

CHAPTER 2.

FOLLICLE AND FIBRE CHARACTERISTICS OF FINEWOOL AND STRONGWOOL MERINOS



Early microscope

CHAPTER 2. FOLLICLE AND FIBRE CHARACTERISTICS OF STRONGWOOL AND FINEWOOL MERINOS

2.1. INTRODUCTION.

There is considerable evidence that the rate of wool growth and the properties of the fibre are determined by -

(i) the number of follicles;

(ii) the number and rate of production of cells in the germinative region of the bulb;

(iii) the proportion of these cells which actually differentiate into cortical cells; and

(iv) the final size and arrangement of these cells in the fibre (Schinckel, 1961; 1962; Short *et al.*, 1965; Fraser, 1965; Black & Reis, 1979; Wilson & Short, 1979; Hynd *et al.*, 1986; Williams & Winston, 1987; Hynd, 1989a).

It is likely that each of these characteristics has a genetic 'maximum', but the actual values expressed are influenced by environmental conditions (Black, 1987). Few measurements have been made of the follicles and fibres of sheep with differing genetic capacity for wool growth (Section 1.5.4.), so there is a lack of conclusive evidence that any of these characteristics, with the exception of follicle density, are causing genetic differences in wool growth.

Measurement of follicle bulb cytokinetics is crucial to the investigation of mechanisms controlling wool growth. Considerable use has been made of agents which arrest dividing cells in metaphase on the assumption that a single, blocked metaphase represents an increase of one cell in the population. The mitotic-inhibiting drug, colchicine, has been used extensively in the study of follicle bulb cytokinetics (Schinckel, 1961, 1962; Short *et al.*, 1965; Fraser, 1965; Wilson & Short, 1979; Hynd *et al.*, 1986; Williams & Winston, 1987; Hynd, 1989a). Colchicine affects cell division by binding tightly to a tubulin dimer, thereby preventing polymerisation of DNA and causing the disappearance of the spindle and accumulation of cells in metaphase. This action is potentially fatal to all actively-dividing tissue and, in the study of follicle bulb cells, has frequently resulted in sheep fatalities when injected intravenously (Schinckel, 1961, 1962; Short *et al.*, 1965; Wilson & Short, 1979).

These effects led to the development of intradermal injection of the drug at lower doses (Hynd *et al.*, 1986; Williams & Winston, 1987).

A major criterion which must be satisfied in order to provide an accurate estimate of the number of dividing cells is that the stages of the cell cycle preceding the metaphase stage must not be affected (Tannock, 1965). This is established by the production of a linear relationship between the number of cells in metaphase and time. The linear accumulation of metaphase cells has been established in studies of follicle bulb cells (Schinckel, 1961; Wilson & Short, 1979; Hynd *et al.*, 1986; Williams & Winston, 1987), although colchicine has been found to depress DNA synthesis of other mammalian tissues at high doses in vitro (Hell & Cox, 1963; Fitzgerald & Brehaut, 1970; Gunther *et al.*, 1976; Kenter *et al.*, 1986; Bhuyan *et al.*, 1987). It is possible that this inhibition may be related to the sensitivity of the tissue studied and there may be a similar inhibition or retardation of the cell cycle by colchicine in the follicle. Thus there is some dissatisfaction with the use of colchicine to study the kinetics of cell division in the follicle bulb (Williams & Winston, 1987; Hynd, 1989a). Hair- and wool-follicle bulb kinetics have also been studied by examining the incorporation of ^{3}H-labelled thymidine in plucked fibres or histological sections following autoradiography (Cattaneo, 1961; Chapman, 1971; Mather & Geo, 1976; Chapman *et al.*, 1980; Weinstein & Mooney, 1980). Disadvantages of autoradiography are that the technique is both time-consuming and expensive and that analysis of labelled mitoses curves does not strictly produce rate of cell production data.

Alternative methods for studies of cell kinetics have been developed including a number of immunohistological techniques. In particular, monoclonal antibodies have been developed to detect the incorporation of the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) into DNA during the S-phase of the cell cycle (Gratzner *et al.*, 1975; Gratzner, 1982; Morstyn *et al.*, 1983; Roberts *et al.*, 1985; DeFazio, *et al.*, 1987; Kikuyama *et al.*, 1988). After a pulse infusion of BrdU, S-phase cells which are labelled and undergo mitotic division cause an increase in the number of labelled cells. This increase is indicative of the rate of cell production since each daughter cells contains half of the original label (Clarke, 1971). The technique has recently been proposed as an alternative to traditional methods for the study of wool-follicle kinetics (Hynd & Everett, 1990; Holle & Birtles, 1990; Adelson *et al.*, 1991)

even though BrdU affects differentiation of cells both in vivo and in vitro (Abbot & Holtzer, 1968; Coleman *et al.*, 1970; Silagi & Bruce, 1970; Miura & Wilt, 1971; Turkington *et al.*, 1971; Weintraub *et al.*, 1972; Wright, 1985; Adelson *et al.*, 1991). Nevertheless, BrdU has no effect on cell proliferation or the cell cycle (Miura & Wilt, 1971) hence, the compound is suitable for use in the study of bulb cell kinetics where cell differentiation is not being examined.

This chapter examines the hypothesis that differences in wool production between sheep housed under similar environmental conditions are due to differences in both the **follicle population** of the sheep and in the functioning of **individual wool follicles.** The aim of this study was to develop and utilise improved methods to extend the investigation of Williams and Winston (1987). Whilst it was recognised that a 'strain'-comparison of sheep reared under environmental conditions does not strictly allow conclusions regarding 'genetic' differences, it was considered that such a comparison would provide a strong indication of where genetic differences may lie. Two strains of Merino sheep were used to generate large differences in wool growth when the animals were exposed to similar environmental and nutritional conditions. The results of the first experiment were then applied to a commercial flock to determine if the relationships were maintained in a commercial situation.

2.2. EXPERIMENTAL PROCEDURE

EXPERIMENT 1.

2.2.1. Experimental design

Six finewool Merinos and six strongwool Merinos were housed in individual pens at Turretfield Research Centre, Rosedale, South Australia and fed a daily ration of lucerne-based sheep pellets (30g / kg liveweight) for 91 days. Water was available *ad libitum.* Once a week, prior to feeding, the animals were weighed using electronic scales (Ruddweigh beef scales), and their rations adjusted according to liveweight. Refusals were collected every two to three days, bulked and then weighed weekly.

Wool growth per unit area of skin was estimated every two to three weeks to determine baseline wool growth rates for each sheep. At the end of the 91 day stabilisation period, during which the sheep were accustomed to the diet and handling procedures, skin

samples were taken to determine the characteristics of the follicle and fibre associated with high levels of wool production as indicated in Figure 2.1. All measurements, unless otherwise specified, were made with a computer-aided image analysis system (Bioquant IV, R & M Biometrics, Tennessee).

2.2.2. Sheep, nutrition and liveweight

The high-wool producing sheep were South Australian strongwool Merino wethers (Bungaree bloodline) randomly-selected from a flock of adult wethers grazing at the Waite Agricultural Research Institute, Adelaide, South Australia. At the commencement of the trial the sheep were aged two to three years. The low-wool producing sheep were finewool Merino wethers (Camden Park bloodline) aged two to three years. These sheep were demonstration sheep on loan from Camden Park to the South Australian Department of Agriculture. Prior to the experiment all sheep were treated for internal and external parasites before being allocated at random to individual indoor pens.

2.2.3. Wool growth

Wool production per unit area of skin was estimated during the two weeks prior to skin sampling (Fig. 2.1) by the tattoo patch technique (Langlands & Wheeler, 1968). At the commencement of the experiment the sheep were tattooed (10 x 12cm) on the right midside region under local anaesthesia (Lignocaine hydrochloride; 20mg/ml; Apex Lab. Pty. Ltd., St Marys, NSW). Every two to three weeks, wool within the tattooed area was closely clipped with small animal clippers and blades (no. 40 blades; Oster Corp., Wisconsin), collected and stored in envelopes. The outline of the tattoo was traced onto a clear acetate sheet and the perimeter of the tracings were measured using an image analysis system (Bioquant IV, R & M Biometrics, Tennessee). The average of three readings was used to obtain the area of the clipped patch.

Greasy wool weight was determined by weighing the wool samples immediately after clipping. The wool samples were then washed in hexane (10 min. x 3; Ace Chemical Co. Camden Park, SA) and hot water (10 min. x 2), to remove grease and suint respectively, and then oven-dried at 60°C for 56 hours. The samples were weighed out of a dessicator

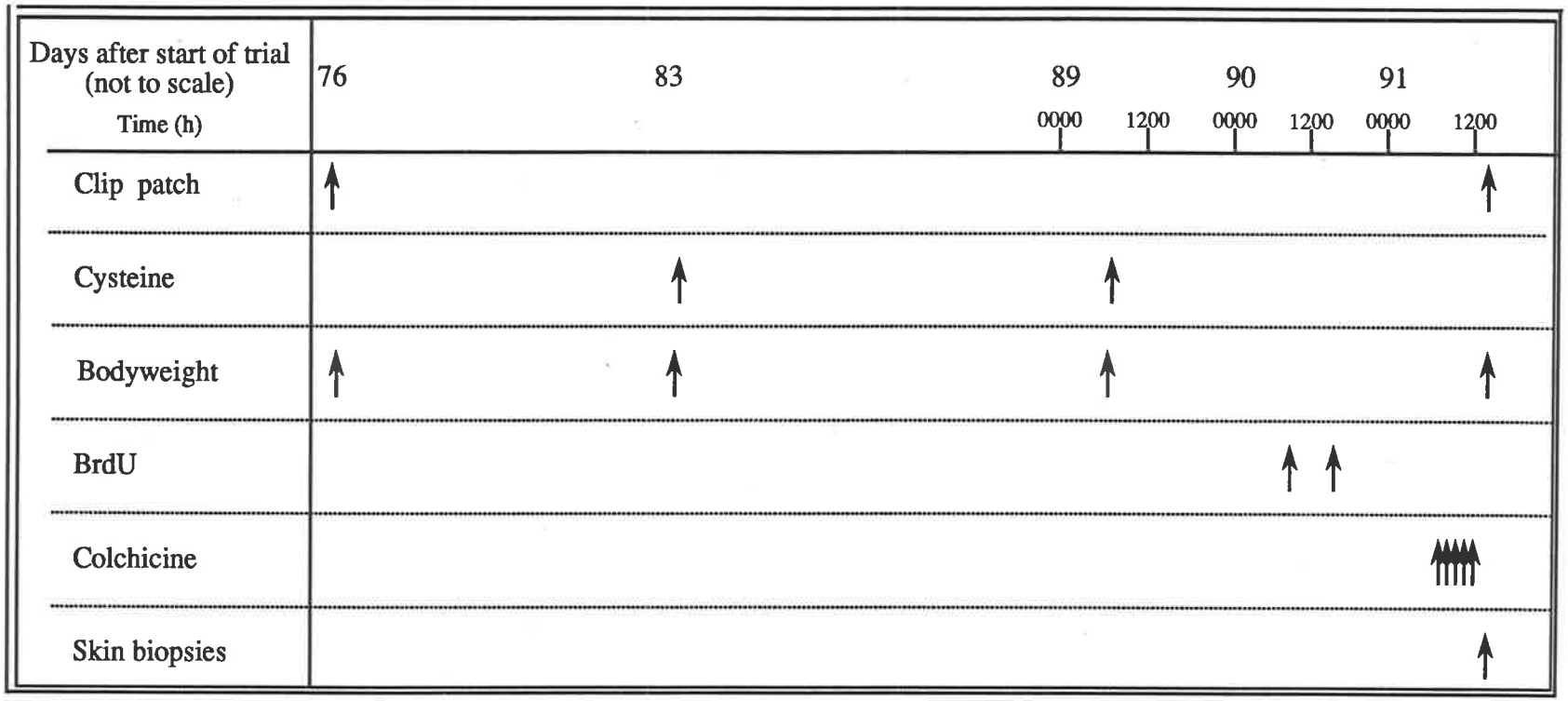


Figure 2.1. Time sequence for sampling tissue and fibre for Experiment 1.

(20 min.) to obtain clean wool weight. Both greasy and clean wool growth rates (g x $10^{-3}/cm^2/d$) were calculated using the area of the clipped patch.

Fibre production per follicle was estimated during the final seven days prior to the end of the trial (Fig. 2.1) by the autoradiographic method of Downes *et al.* (1967). L-[^{35}S]-cysteine hydrochloride (5μCi/ml, 114mCi/mmol; Amersham Australia, North Ryde, NSW) was injected intradermally (0.4ml) into a site adjacent to the midside patch seven days prior and again immediately before the end of each experiment. The radiolabelled wool was clipped off close to the skin 30 days after the final injection. The wool was washed in hexane (5 min. x 3; Ace Chemical Co. Camden Park, SA) and hot water (5 min.), stained in saturated picric acid (2 min.), rinsed in hot water and blotted dry. At least 75 fibres per sheep were mounted on microscope slides coated with 20% PVP 40 (polyvinyl pyrrolidone; Sigma, St Louis, Mo.). The slides were exposed to X-ray film (Agfa-Gevaert, Belgium) and stored at 4°C for 7 days. The film was processed as follows; placed in 20% developer solution (4 min.; Phenisol X-ray Developer; Ilford Pty Ltd, Mt Waverly, Vic.), rinsed in water, 20% fixer solution (4 min.; Hypam X-ray Rapid Fixer; Ilford Pty Ltd, Mt Waverly, Vic.) and rinsed in water for 15 to 20 minutes. The films were air-dried before being placed on the slides with D.P.X. (Gurr; BDH Chemical Aust. Pty Ltd; Kilsyth, Vic). The film was aligned with the fibres using the radioactivity markings.

The volume fibre produced was determined using the computer-aided image analysis system (Bioquant IV, R & M Biometrics, Tennessee). The length of the fibre between to two radiation marks was measured at a magnification of 155 times (or 95 times if the fibres were too long to fit on the video screen). The diameter of the fibre was measured (1539 times magnification) at five sites equidistant along the fibre between the radiation marks from which the mean fibre diameter was estimated. The volume of fibre cortex produced per hour was estimated using Equation 2.1, with the assumption made that the fibre cuticle was 0.5μm thick (Wilson & Short, 1979).

Equation 2.1.

$$\text{Volume of cortex produced per unit time } (\mu m^3/h) = \pi \times \frac{[\text{Mean fibre diameter} - 1]^2}{4} \times \frac{\text{Fibre length}}{\text{number of hours}}$$

2.2.4. Histology

Four skin biopsies for general histology were sampled from adjacent to the midside patch. After local anaesthesia with Lignocaine hydrochloride (0.5ml of 20mg/ml; Apex Lab. Pty Ltd, St Marys, NSW), biopsies were taken with a trephine (1cm diameter), removed with surgical forceps and scissors (Carter & Clarke, 1957) and placed in 10% buffered formalin ($NaH_2PO_4.2H_2O$* [4g], Na_2HPO_4* [6.5g], H_2O [900ml], formalin† [90% (w/w), 100ml]). Excess wool was removed from the sample after 7 days of fixation and the sample was then stored in 70% (v/v) ethanol until processed and sectioned as described in Appendix 1.1.

2.2.4.1. Follicle density

Skin sections were cut (10μm) transverse to the follicle at the level of the sebaceous gland, deparaffinised, rehydrated and stained with methylene blue (Appendix 1.2.1. and 1.2.2.b). Follicle density was determined at a magnification of 425 times and corrected for tissue shrinkage (estimated by measuring the skin sample post-sampling and relating this to the original 0.78cm^2 biopsy area) according to equation 2.2.

Equation 2.2.

$$\text{Follicle density } (mm^{-2}) = \frac{\text{Number of follicles}}{\text{Known area}} \times \frac{\text{Area of sample}}{78.54}$$

2.2.4.2. Volume of germinative tissue

The germinative area of the bulb was defined as the total bulb region which terminated one cell width above the papilla (Plate 2.4; Fraser, 1965). Serial sections were cut (6μm) longitudinal to the follicle, deparaffinised, rehydrated and stained with haematoxylin and eosin (Appendix 1.2. and 1.2.2.a.). The area of the germinative tissue of each bulb was measured and multiplied by the section thickness (6μm) to derive the volume of germinative tissue in a bulb section. The volume of germinative tissue in a whole bulb was then estimated by reconstruction of 25 bulbs per sheep (1019 times magnification).

The total germinative volume of skin was estimated from the product of the follicle density and the average volume of germinative tissue in each bulb.

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* AR grade products from BDH Chemicals, Kilsyth, Vic.

† LR grade product from Ajax Lab. Pty, Ltd, St Marys, NSW

2.2.4.3. Number and volume of bulb cells

Skin sections were cut longitudinal to the follicle at both 4 and 6μm and every tenth and seventh section respectively, was retained. This, together with the criterion that only those bulbs containing dermal papilla were measured, prevented measurement of the same bulb twice. The sections were deparaffinised, rehydrated and stained with haematoxylin and eosin (Appendix 1.2. and 1.2.2.a). The 'true' number of cells in the bulb was estimated by the technique of Abercrombie (1946). For each section thickness, the number of cells in the germinative region of the bulb was counted and the area measured (magnification of 1019 times) in 200 follicle bulbs which contained dermal papilla cells. These bulb sections were selected to ensure that the counted cells were actually germinative cells, rather than inner root sheath cells or cells in the pre-keratinisation region. For each sheep, the volume and 'true' number of cells per bulb was estimated as described in Equations 2.3 to 2.6.

Equation 2.3.

'True' no. of cells in a 2μm section

= No. cells in a 6μm section - No. cells in a 4μm section

Equation 2.4.

Mean volume of 2μm section (μm^3)

= Mean bulb area of the 4 & 6μm sections x 2 (μm)

Equation 2.5.

$$\text{Mean volume of bulb cells } (\mu m^3) = \frac{\text{Mean section volume [Eq.2.4]}}{\text{'True' no. of cells in section [Eq.2.2]}}$$

Equation 2.6.

$$\text{Mean number of cells per bulb} = \frac{\text{Mean bulb volume [Sec. 2.2.4.2]}}{\text{Mean volume of bulb cells [Eq.2.5]}}$$

2.2.4.4. Cell kinetics

The rate of bulb cell production was determined by two methods; a stathmokinetic method described by Hynd *et al.*, (1986) and an immunohistochemical technique as developed by Hynd and Everett (1990).

a) Stathmokinetic technique

Colchicine (Sigma Chemical Co., St. Louis, Mo. USA) was injected into the dermis (0.1ml; 500µg/ml saline) at five sites on the left midside of each sheep. Skin biopsies were taken as described in Section 2.2.4. immediately after the first injection and then hourly for four hours (Fig. 2.1.). The tissue samples were fixed in Zenker-acetic acid mixture for four hours (5% [w/v] $HgCl_2$*;2.5% [w/v] $K_2Cr_2O_7$† ; 5% [v/v] glacial acetic acid# added immediately before the skin sample), washed in running water overnight, placed in 50% (v/v) ethanol and 1% (w/v) KI† for 12 hours, 70% (v/v) ethanol and 1% (w/v) KI† for 12 hours and then stored in 70% (v/v) ethanol. Samples were embedded in paraffin and sectioned as described in Appendix 1.1.

Sections were cut (4µm) longitudinal to the follicle, deparaffinised, rehydrated and stained with haematoxylin and eosin (Appendix 1.2.1. and 1.2.2.a.). Random bulb counts were made by recording the frequency of follicles having 0,1,2,3,....n mitotic nuclei per bulb section in 200 to 300 bulbs (Schinckel 1961). Data are presented as medians since the frequency distribution of cell counts was not always normal. Rate of cell production was determined by the regression coefficient and the Students t-test was used to determine if the regression coefficient differed between strains.

b) Is there a lag period associated with colchicine injection?

Tissue samples were taken from a single strongwool Merino sheep injected intradermally with 20µg (200µg/ml) of colchicine. After colchicine administration, biopsies were taken at 15 minute intervals for 90 minutes and then every 30 minutes until 180 minutes after injection. The skin was then fixed, processed, sectioned and stained as described in section 2.2.4.4.a.

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* AR grade product supplied by BDH Chemicals, Poole, England

† AR grade product supplied by Ajax Lab., Sydney, NSW

\# Supplied by CSR Chemicals, Rhodes, NSW

c) Immunohistochemical determination

5-Bromo-2'-deoxyuridine (BrdU; Sigma Chemical Co., St. Louis, Mo. USA) was injected into the jugular vein (200mg in 20ml saline) and skin biopsies taken from the upper edge of the tattoo patch as described in section 2.2.4., four and eight hours after injection. The samples were fixed in Zenker-acetic acid fixative for one hour and processed as described above (section 2.2.4.4.a). BrdU was detected after deparaffinisation and rehydration using histochemical techniques modified from DeFazio *et al.*, (1987), Hynd and Everett (1990) and Holle and Birtles (1990) as described in Appendix 1.2.3.

The percentage of mitotic cells labelled with BrdU was estimated, as a prerequisite for the determination of cell production rate (Clarke, 1971). In both the four and eight hour samples, the number of cells labelled with BrdU were counted in at least 180 bulbs sectioned close to the midline. This number of bulbs gave a coefficient of variation (CV) between 23% and 33% (depending on the sheep), whereas counting 240 bulbs gave a CV of approximately 27%. Thus, it was concluded that given the variation of the size of the bulb and the number of cells in the bulb within a single sheep, counting 180 bulbs was sufficient to give a representative number of cells per bulb. The increase in the number of labelled cells over the four hour sampling period was used to estimate cell production rate (Equation 2.7). The average turnover time of the bulb was then estimated using Equation 2.8.

Equation 2.7

$$\text{Cell production rate (bulb}^{-1}\text{ h}^{-1}\text{)} = (\text{No. cells labelled in 8h sample} - \text{No. cells labelled in 4h sample}) / 4 * \frac{\text{Total bulb volume}}{\text{Mean section volume}}$$ [Section 2.2.4.2]

Equation 2.8

$$\text{Bulb turnover time (h)} = \frac{\text{Total number of cells in bulb [Eq 2.6]}}{\text{Cell production rate[Eq 2.7]}}$$

2.2.5. Cortical cells

Cortical cells were liberated from the fibres with formic acid as described below. This method was used after the analyses and comparisons were made with other techniques (Appendix 2.). Wool from the midside patch, used for the estimation of clean wool growth rate, was washed in a further two changes of hexane (5 min.) and three changes of warm

water (5 min.), dehydrated in ethanol and air dried. Three samples (approximately 5mg each) were placed in 90% formic acid (20ml) at 60°C for 24 hours, centrifuged (2000*g*, 4°C; MSE Mistral 4L), then the formic acid was removed with a pasteur pipette and the wool stored in water overnight. The samples were ultrasonicated at 50-80W (Labsonic 1510, B.Braun) for at least 15 minutes until the majority of fibres had been dispersed. The three samples were combined and 3 drops of cell suspension were placed onto microscope slides coated with Poly-L-Lysine (Sigma Diagnostics., St. Louis, Mo.) and air-dried. The cells were stained with saturated picric acid (5 min.), rinsed in water, dehydrated through 80% (v/v) ethanol (10 sec.), absolute ethanol (2 min.), Safsolvent (5 min.) and mounted on a glass slide with D.P.X. (Gurr; BDH Chemicals, Kilsyth, Vic.).

The length and width of the cortical cells were measured at 1188 times magnification and the volume of individual cells was determined using the formula proposed by Short *et al.*, (1965). This assumes that the cortical cells are shaped like two cones placed base to base so that volume can be calculated using Equation 2.9. The number of cortical cells produced per hour (Equation 2.10) and the proportion of bulb cells contributing to fibre production (efficiency, Equation 2.11.) were calculated as described by Wilson and Short (1979) and Hynd (1989a).

Equation 2.9.

$$\text{Cortical cell volume } (\mu m^3) = 2 \times \frac{\text{Cell length}}{2} \times \frac{1}{3} \times \frac{\pi\,[\text{Cell width}]^2}{4}$$

Equation 2.10.

$$\text{Cortical cells produced per hour } (h^{-1}) = \frac{\text{Fibre production rate [Sec.2.2.3.]}}{\text{Cortical cell volume [Eq.2.9]}}$$

Equation 2.11.

$$\text{Efficiency} = \frac{\text{No. cortical cells produced per hour [Eq. 2.10]}}{\text{No. mitotic cells produced per hour [Eq.2.7]}}$$

EXPERIMENT 2.

2.2.6. Experimental design

Twenty four ewes aged 4 years were selected from the control and special stud flocks of ANAMA Merino Stud, located near Clare, South Australia. The details concerning these flocks and their response to breeding using WOOLPLAN selection indices have been published elsewhere (Hynd *et al.*, 1989; Hawker *et al.*, 1991). Briefly, sheep at the ANAMA

stud have been selected with the aid of a WOOLPLAN selection index, combining clean fleece weight, fibre diameter and hogget liveweight, since 1978. Simultaneously, a randomly-selected control flock with minimum culling was maintained. Twelve ewes from each flock for the present experiment were selected from 38 control ewes and 39 stud ewes, according to a greasy fleeceweight (GFW) and liveweight (BW) index (GFW x 10 / BW). The ewes with the highest index from the stud flock and the lowest from the control flock were chosen and transported to the Waite Institute, Adelaide, South Australia.

The animals were housed indoors during the 98 day stabilisation period and fed a maximum of 2.2kg per day, based on 30g/kg liveweight per day, of grain-based sheep pellets (Milling Industries, Murray Bridge, SA) for 80 days. This was decreased to 21g/kg liveweight per day for 61 days, since most sheep failed to consume the ration allocated to them. Water was available *ad libitum*. Animals were weighed weekly using electronic scales (Ruddweigh beef scales), and their rations adjusted according to their liveweights. Refusals were collected and weighed every two to three days.

Wool growth, volume of germinative tissue (magnification 1188 times) and follicle density (magnification 259 times) were determined as described in sections 2.2.3., 2.2.4.1. and 2.2.4.2., with minor amendments. Firstly, the ^{35}S-labelled wool was clipped from the sheep 15 days after the final injection for the estimation of fibre growth and, secondly, the volume of germinative tissue was estimated from skin sections cut at 8μm rather than 6μm. Wool growth per unit area of skin and fibre cortex production per individual follicle were determined over the last 20 days and 5 days, respectively.

2.2.7. Statistical analysis

The mean of and standard error of the mean for the various characteristics within each group of sheep were routinely calculated. The students t-test was then used to compare the characteristics between strains. Results were considered significantly different when $P < 0.05$.

Analysis of variance statistics were performed with the Super ANOVA computer software package (1989-1990, Abacus Concepts, Inc. Berkeley, California) and was used to test both the effects of time and strain on the percentage of BrdU-labelled mitotic cells and

the effect of colchicine and BrdU and strain on the number of cells produced in the bulb. Results were considered significant when $P < 0.05$.

Simple correlation coefficients were estimated with GENSTAT 5 (1988, Lawes Agricultural Trust, Rothamsted Experimental Station). The coefficients were used to analyse wool growth and the associated characteristics and were considered significantly correlated when $P < 0.05$.

Stepwise linear regression was performed in two stages with GENSTAT 5 (1988, Lawes Agricultural Trust, Rothamsted Experimental Station) to derive associations between the measured characteristics and both wool production per unit area and fibre cortex produced per follicle . In this analysis, the parameter estimates of the regression model and their standard errors were examined and the factors with the smallest non-significant t-value were eliminated until no more parameters could be removed from the equation.

2.3. RESULTS

EXPERIMENT 1.

2.3.1. Wool growth, liveweight and feed intake

There was a highly significant difference in wool growth rate between the two strains of Merino sheep maintained under similar environmental conditions. The strongwool Merinos grew 2.5 times more wool per unit area of skin and 3.5 times more fibre cortex per follicle than the finewool Merinos (Table 2.1.). The difference in fibre volume was largely due to variation in fibre diameter rather than in length growth rate (Table 2.1.).

Table 2.1. Wool production per unit area of skin (wool), fibre diameter, length growth rate and the volume of fibre cortex produced per follicle (fibre cortex) for finewool and strongwool Merinos (Means with s.e.m. in parentheses)

	Finewool*	Strongwool*	*P* =
Wool (mg / cm^2 / day)	0.52 (0.06)	1.29 (0.05)	0.001
Fibre diameter (μm)	17.8 (0.52)	29.8 (0.66)	0.001
Fibre length growth rate (μm / day^{-1})	324 (14.5)	388 (17.1)	0.025
Fibre cortex ($\mu m^3 \times 10^3 h^{-1}$)	3.0 (0.30)	10.5 (0.59)	0.001

* Wool production per unit area of skin was measured over the last 14 days of the trial period and other measurements were made over the last seven days of the trial

The sheep consumed the majority of their ration for the duration of the experiment (Appendix 3.1.2), with the exception of three finewool Merinos. The average liveweight of the finewool Merinos did not change during the stabilisation period (Section 2.2.1), whereas the average liveweights of the strongwool Merinos increased significantly ($P < 0.005$; Table 2.2.). The feed intake of the sheep increased in relation to liveweight throughout the stabilisation period, nevertheless, during the final 14 and seven day periods when wool growth estimates were made, feed intake per kilogram liveweight did not differ significantly between the two strains of sheep (Table 2.2.). It was necessary to feed the sheep on a per unit liveweight basis due to the large difference in liveweight between the two strains. As the strongwool Merinos were significantly heavier, they had a higher total daily intake than the finewool Merinos, thus there only tended to be a difference in the efficiency of wool production per unit feed intake between the two strains (Table 2.2.).

Table 2.2. Liveweights at the commencement and conclusion of the trial, feed intake during the final wool measurement periods and efficiency of wool production per unit feed intake of finewool and strongwool Merinos (Means with s.e.m. in parentheses)

	Finewool	Strongwool	*P<*
Initial liveweight (kg)	41 (4.6)	54 (1.3)	0.025
Final liveweight (kg)	45 (3.8)	64 (2.2)	0.005
Feed intake during final 14 days (g / kg /d)	26 (1.1)	27 (0.7)	nsd†
Feed intake during final 7 days (g / kg / d)	27 (1.4)	26 (1.6)	nsd†
Efficiency of wool growth (mg / kg)	0.53 (0.098)	0.79 (0.102)	0.088

† no significant difference

The primary objective of the present experiment, that non-nutritional wool growth differences should be generated, was achieved since neither wool growth per unit area of skin ($r = 0.27$) nor cortex production ($r = 0.11$) were correlated with feed intake per unit liveweight during the final 14 days and 7 days respectively. The two measurements of wool production and total daily intake (i.e. when liveweight was not taken into account) were correlated ($P < 0.01$; $r = 0.78$, for wool growth per unit area of skin; and $r = 0.80$, for cortex production).

2.3.2. Skin and follicle characteristics

The strongwool Merinos had a significantly lower follicle density and greater mean volume of germinative tissue in the bulb than the finewool Merinos (Table 2.3). These differences are evident in Plates 2.1.a.,b. and 2.2.a.,b. The total germinative volume of skin, derived from the product of bulb volume and density, was significantly greater ($P < 0.002$) in the strongwool Merinos than the finewool Merinos. This was despite a significant negative relationship between density and volume of germinative tissue in the bulb (Fig. 2.2.; $r = -0.79$; $P < 0.01$). The total germinative volume of the skin (x) was highly correlated with wool production per unit area of skin (y) as is evident in Figure 2.3. and Table 2.7, and the relationship remained significant between sheep-within strains. Furthermore, the ß-coefficient was similar for both regression equations (Equations 2.12a, b).

Finewool Merinos (Equation 2.12a)

$$y = 0.03 + \mathbf{0.011x} \qquad (r = 0.76),$$

Strongwool Merinos (Equation 2.12a)

$$y = 0.6 + \mathbf{0.010x} \qquad (r = 0.90).$$

There was no difference between strains in the number and volume of bulb cells nor in the number of potentially-mitotic cells per unit area of skin. The number and volume of individual bulb cells tended to be higher in the strongwool Merinos, associated with the larger bulb volume (Table 2.3).

Table 2.3. Follicle density, volume of the germinative tissue in the bulb (bulb volume), number and volume of cells in the germinative region, the number of potentially-mitotic cells per unit area of skin (pot. mitotic cells) and total germinative volume of the skin (total germ. volume) of finewool and strongwool Merinos (Means with s.e.m. in parentheses)

	Finewool	Strongwool	*P* =
Follicle density (mm^{-2})	52.3 (3.99)	37.2 (3.22)	0.012
Bulb volume ($\mu m^3 \times 10^4$)	8.6 (0.92)	19.2 (1.35)	0.001
Bulb cell number	535 (87.6)	830 (165.2)	0.184
Cell volume (μm^3)	176 (26.5)	261 (37.3)	0.094
Pot. mitotic cells ($mm^{-2} \times 10^3$)	27.2 (4.37)	31.5 (7.65)	0.678
Total germ. volume ($\mu m^3 \times 10^5\ mm^{-2}$)	44 (4.3)	70 (4.7)	0.002

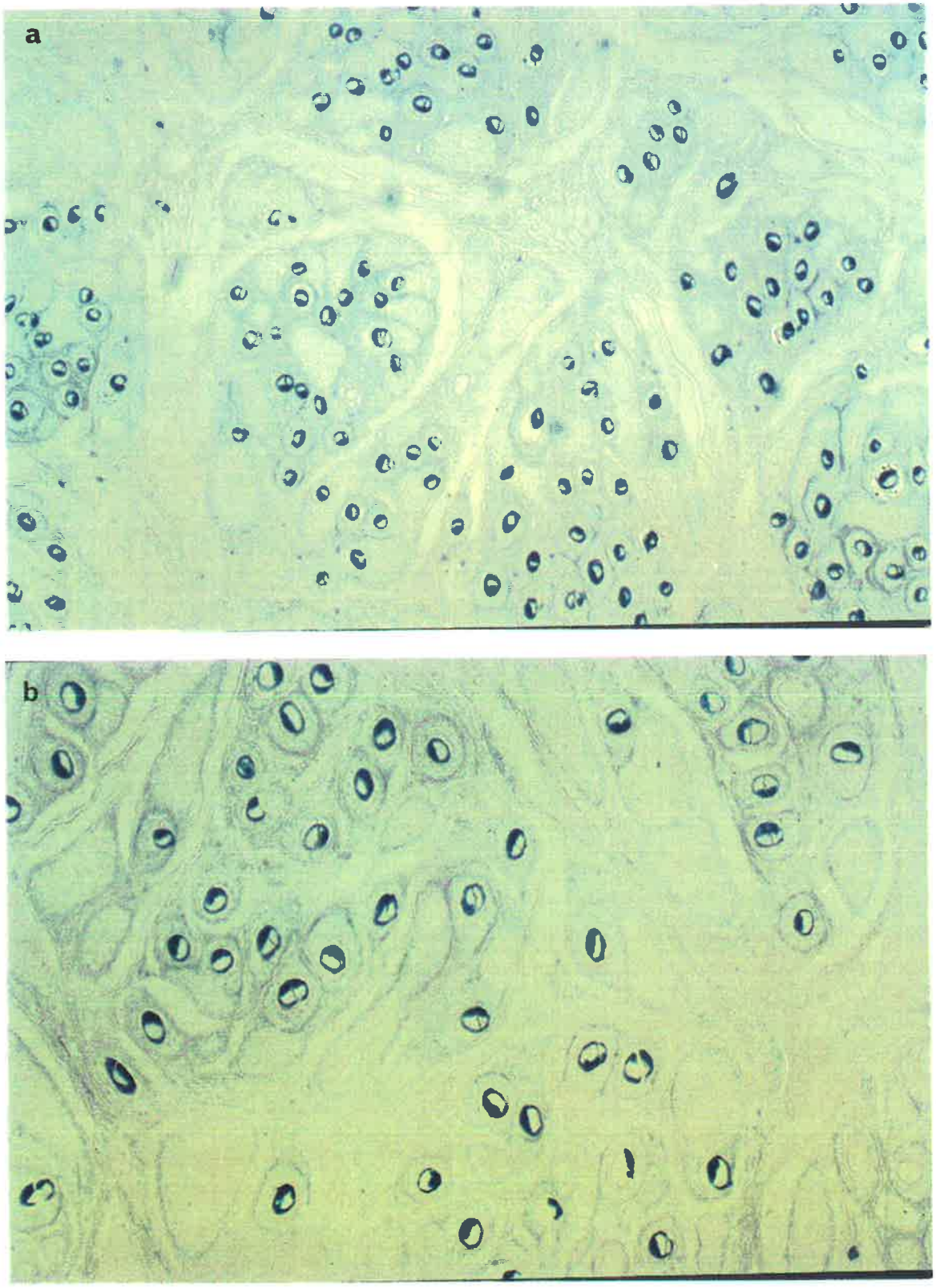

Plate 2.1. Transverse sections through the skin at the level of the sebaceous glands showing the fibres after staining with methylene blue (Clarke & Maddocks, 1965); Magnification: 425x.

a) Skin sample from a finewool Merino (note the high follicle density)

b) Skin sample from a strongwool Merino (note the low follicle density).

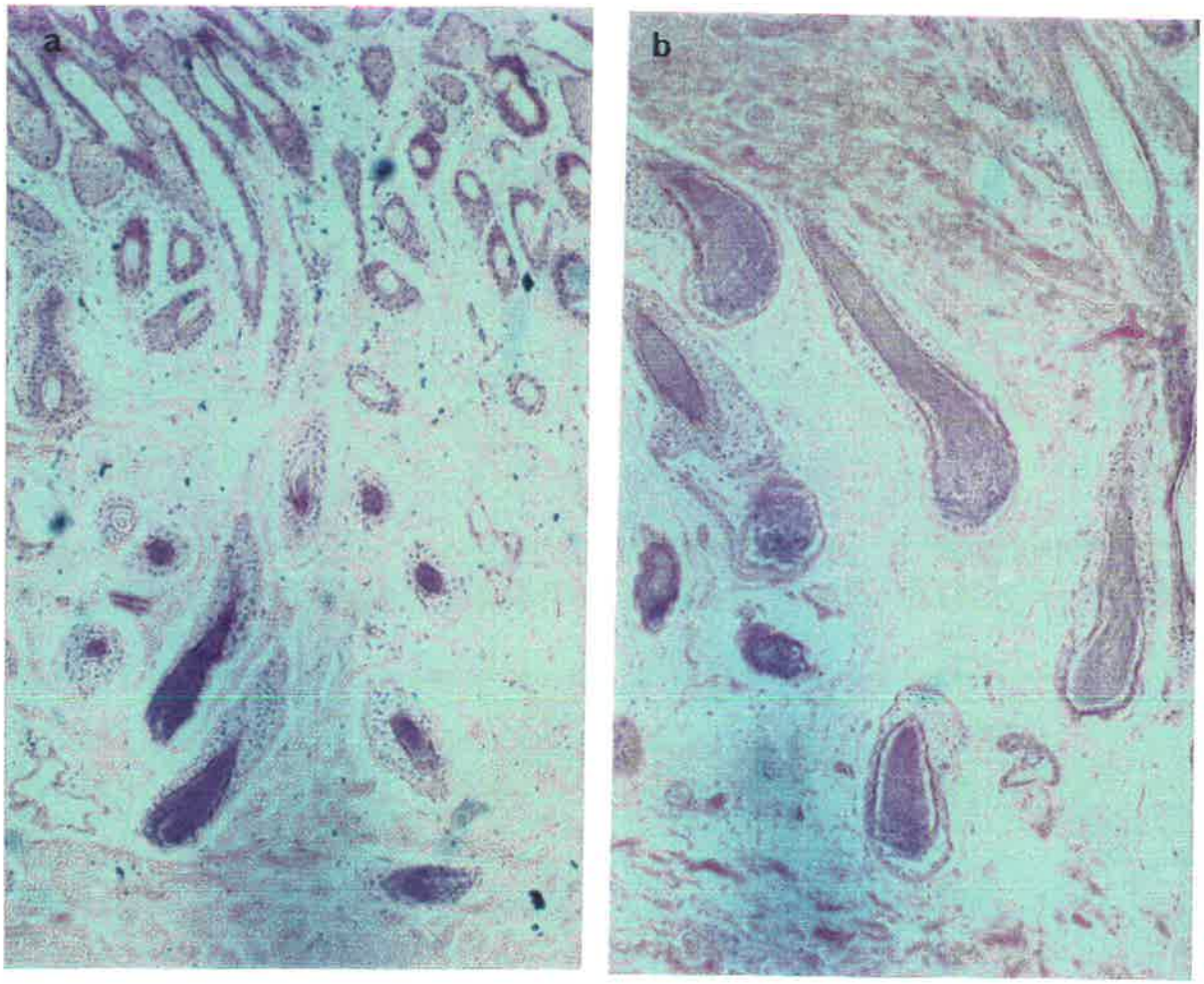

Plate 2.2. Longitudinal sections through skin stained with haematoxylin and eosin (Appendix 1.2.2.); Magnification 425x.

a) Skin sample from a finewool Merino

b) Skin sample from a strongwool Merino.

Note the diference between the strains in the size of the bulbs and the total proportion of skin to which the bulbs are contributing

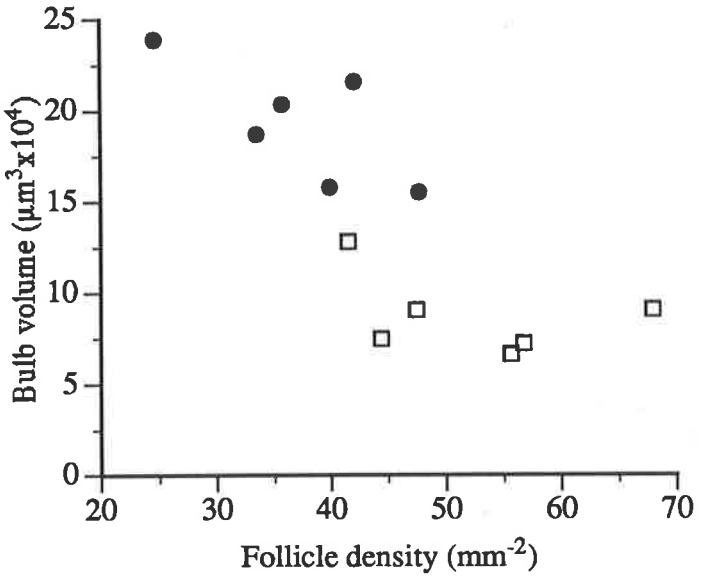

Figure 2.2. The relationship between follicle density and volume of germinative tissue in the follicle bulb of finewool (□) and strongwool (●)Merinos. (r = -0.79; P < 0.01)

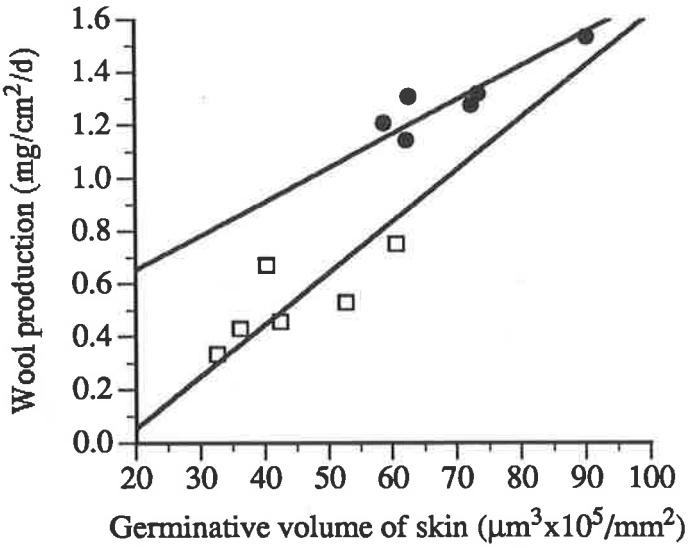

Figure 2.3. The relationship between the total volume of germinative tissue in the skin and wool production per unit area of skin of finewool (□) and strongwool (J) Merinos. (r = 0.91; P < 0.01)

2.3.3. Cell proliferation in the follicle bulb

2.3.3.1. Colchicine versus Bromodeoxyuridine (BrdU)

The injection of colchicine arrested bulb cell nucleii in metaphase as expected (Plate 2.3) but the increase in the number of arrested cells in the finewool Merinos was not linear from 0 to 4 hours (Appendix 3.1.3.a). The correlation coefficients between time and the number of mitotic cells per bulb per 4μm section were lower when the 0 hour samples were included than when the regression was calculated from 1 to 4 hours (Appendix 3.1.3.b). Thus, regression coefficients were estimated from 1 hour after colchicine administration for both strains of Merinos, and these values were used for the comparison with the BrdU method.

The lag time observed for the arrest of cells in metaphase in the finewool Merinos was investigated by studying the effects of colchicine on the arrest prior to the usual first sampling at one hour after colchicine administration (Section 2.2.3.4.b). There was a lag in the accumulation of cells in metaphase for the first 15 minutes after which there was a rapid accumulation in the number of cells arrested in metaphase (Fig. 2.4). The rate of cells entering mitosis then became linear between 45 and 60 minutes after colchicine administration.

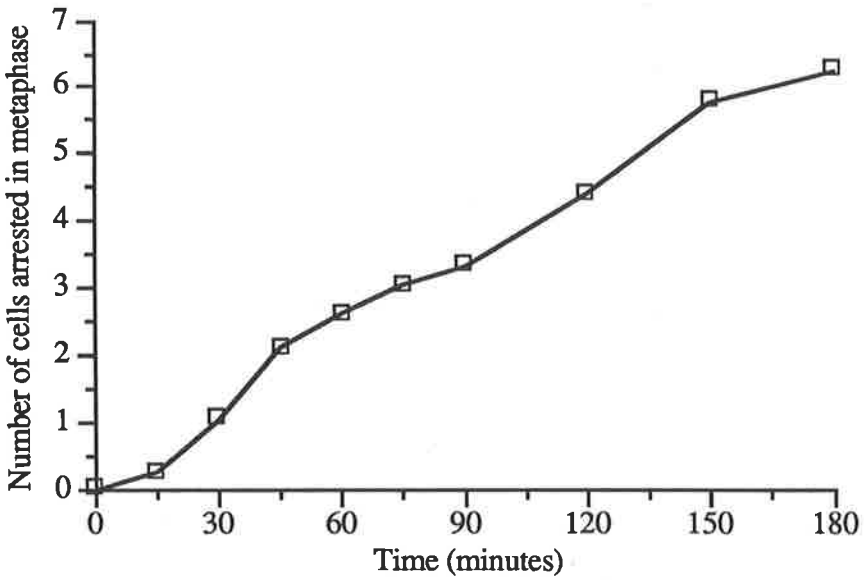

Figure 2.4. The number of cells arrested in metaphase (number of mitotics per 8μm bulb section) in the follicle bulbs of a single strongwool Merino over time (Section 2.2.4.4.b.)

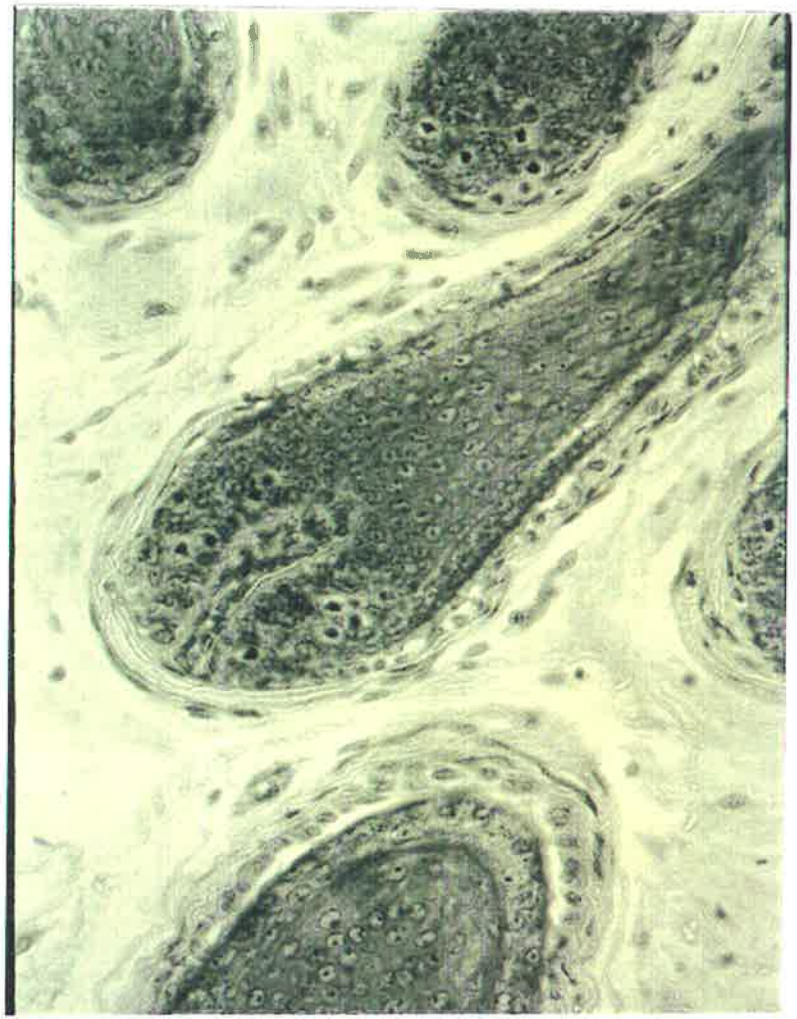

Plate 2.3. Longitudinal section through skin sampled four hours after colchicine injection. Note the even distribution of the cells arrested in metaphase and the absence of any cells progressing beyond metaphase.

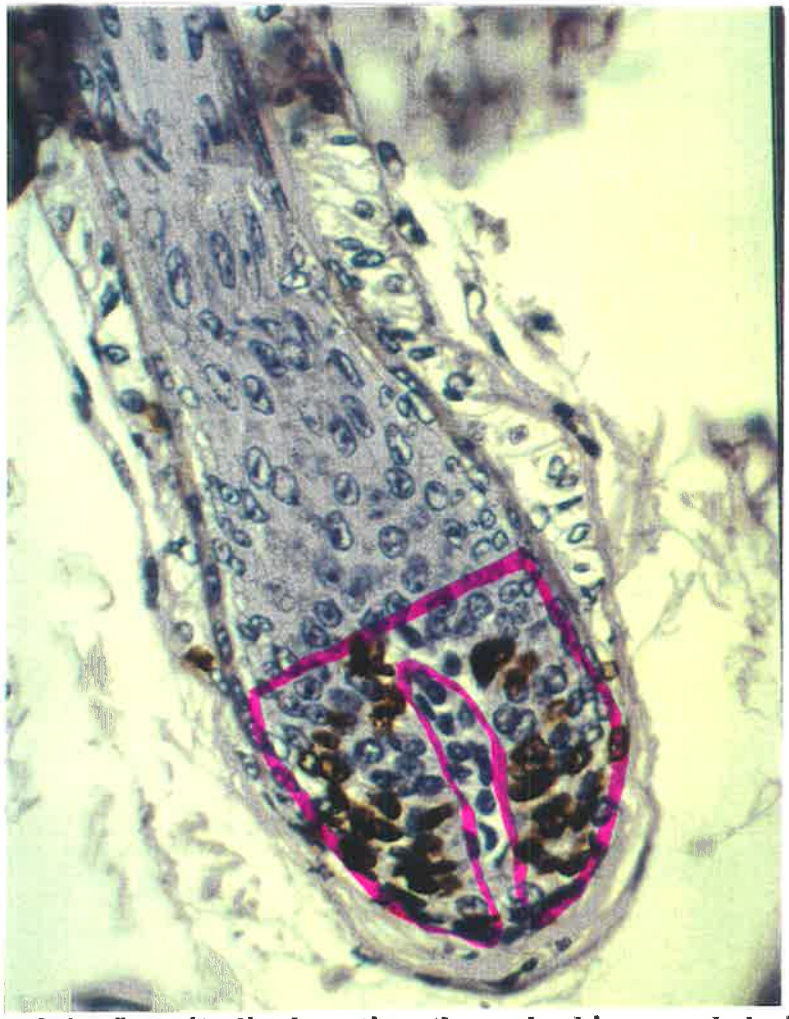

Plate 2.4. Longitudinal section through skin sampled eight hours after a systemic bolus dose of BrdU. The cells labelled with BrdU have been detected with the method described in Appendix 1.2.3. The delineation of the area of germinative tissue in the bulb is outlined with pink.

The cells which incorporated BrdU into their DNA stained brown with the immunohistochemical technique described in Section 2.2.3.4.c (Plate 2.4) and were evenly distributed throughout the bulb region. The proportion of cells undergoing mitosis which were labelled with BrdU was close to 100% (Table 2.4). The analysis of variance indicated that there was a significant difference between strains in the number of BrdU-labelled cells in mitosis ($P = 0.002$). There was no significant difference between the 0 and 8 hour samples ($P = 0.365$), nor was there an interaction between strain and time ($P = 0.509$). It was considered that at least 95% labelling of cells in mitosis gives an adequate estimate of cell production rate.

Table 2.4. Percentage of mitotic cells labelled with BrdU in skin samples taken 4 and 8 hours after infusion of BrdU (Mean of strain with s.e.m. in parentheses)

	4 hour	8 hour
Finewool Merino	98 (1.0)	100 (0.3)
Strongwool Merino	95 (1.3)	95 (1.4)

The analysis of variance statistics indicated there was a significant difference between the stathmokinetic and immunohistochemical methods used for the estimation of cell division rate ($P < 0.001$) and between the two strains ($P = 0.049$). There was no interaction between strain and technique ($P = 0.430$). The immunohistochemical technique gave a higher estimation of cell production rates (Table 2.5a,b.).

Table 2.5.a. The number of cells produced in the follicle bulbs of finewool Merinos estimated using colchicine and BrdU

Sheep number Finewool	Colchicine (No. mitotics/4μm section/h)	BrdU (No. cells produced/4μm section/h)
0	1.08	0.99
62	0.86	1.84
753	0.66	2.40
1187	0.81	1.02
1237	0.65	1.95
1242	0.56	2.63
Mean (sem)	0.77 (0.077)	1.81 (0.279)

Table 2.5.b. The number of cells produced in the follicle bulbs of strongwool Merinos estimated using colchicine and BrdU

Sheep number Strongwool	Colchicine (No. mitotics/4μm section/h)	BrdU (No. cells produced/4μm section/h)
820	1.31	1.51
821	1.18	4.05
844	0.79	4.39
851	1.18	2.46
875	1.49	1.62
877	0.97	1.98
Mean (sem)	1.15 (0.101)	2.67 (0.511)

In summary, the BrdU method was used to determine cell production rate for the analysis of factors associated with wool growth since it satisfied the pre-requisite of 100% of mitotics labelled during the period of estimation. Conversely, the colchicine method did not meet one of the important criteria for its use, namely that there should be linearity in the metaphase arrest curves over time.

2.3.3.2. Cell kinetics

The strongwool Merinos produced significantly more cells per bulb per hour than the finewool Merinos ($P = 0.044$; Table 2.6.). The rate of cells produced per bulb was correlated with wool production per unit area of skin ($r = 0.70$; $P < 0.02$) but not with production of individual fibres ($r = 0.51$, $P < 0.1$; Table 2.7.). The lack of significance in the latter was due to the low number of sheep and the high standard errors of the means. There was no difference between strains in the time for the population of cells to replace itself, i.e. bulb turnover time, although the strongwool Merinos tended to have a shorter turnover time than the finewool Merinos (Table 2.6.). This lack of significance in the trend was due to a large standard error which resulted from bulb turnover times ranging from 15 to 68 hours in the finewool Merinos and 11 to 37 hours in the strongwool Merinos (Appendix 3.1.1.a. & b.).

Table 2.6 The rate of cell production in wool follicle bulbs (cell production) and the average turnover time of the bulb of finewool and strongwool strains of Merinos (Means with s.e.m. in parentheses)

	Finewool	Strongwool	P =
Cell production (bulb^{-1} h^{-1})	18.3 (2.18)	37.7 (7.51)	0.044
Turnover time (h)	31.8 (7.50)	24.0 (4.08)	0.395

Table 2.7. Relationships between follicle and fibre characteristics and both wool production per unit area (wool production) and the production of fibre cortex per follicle (fibre volume) (Correlation coefficients)

	Wool production	Fibre volume
Fibre volume	0.92 ***	-
Density	-0.48	-0.719 **
Bulb cell volume	0.34	0.55
Bulb cell number	0.58 *	0.42
Germinative volume of individual bulbs	0.86 ***	0.95 ***
Germinative volume of skin	0.91 ***	0.75 ***
Cell production rate	0.70 **	0.51
Turnover time	-0.26	-0.20
Potentially-mitotic cells	0.35	0.07
Cortical cell volume	0.64 *	0.58 *
Cortical cell number	0.84 ***	-
Cell production rate : Cortical cell number	0.39	-

* $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$

2.3.4. Cortical cell production

There was a lot of variation in cortical cell size within a sheep (Plate 2.5a), and the strongwool Merinos tended to have cortical cells with larger average volume ($P = 0.059$; Table 2.8.) than the finewool Merinos. This was associated with a significantly greater cortical cell diameter in the strongwool Merinos rather than cell length differences. The later was not significantly different between strains (Table 2.8.).

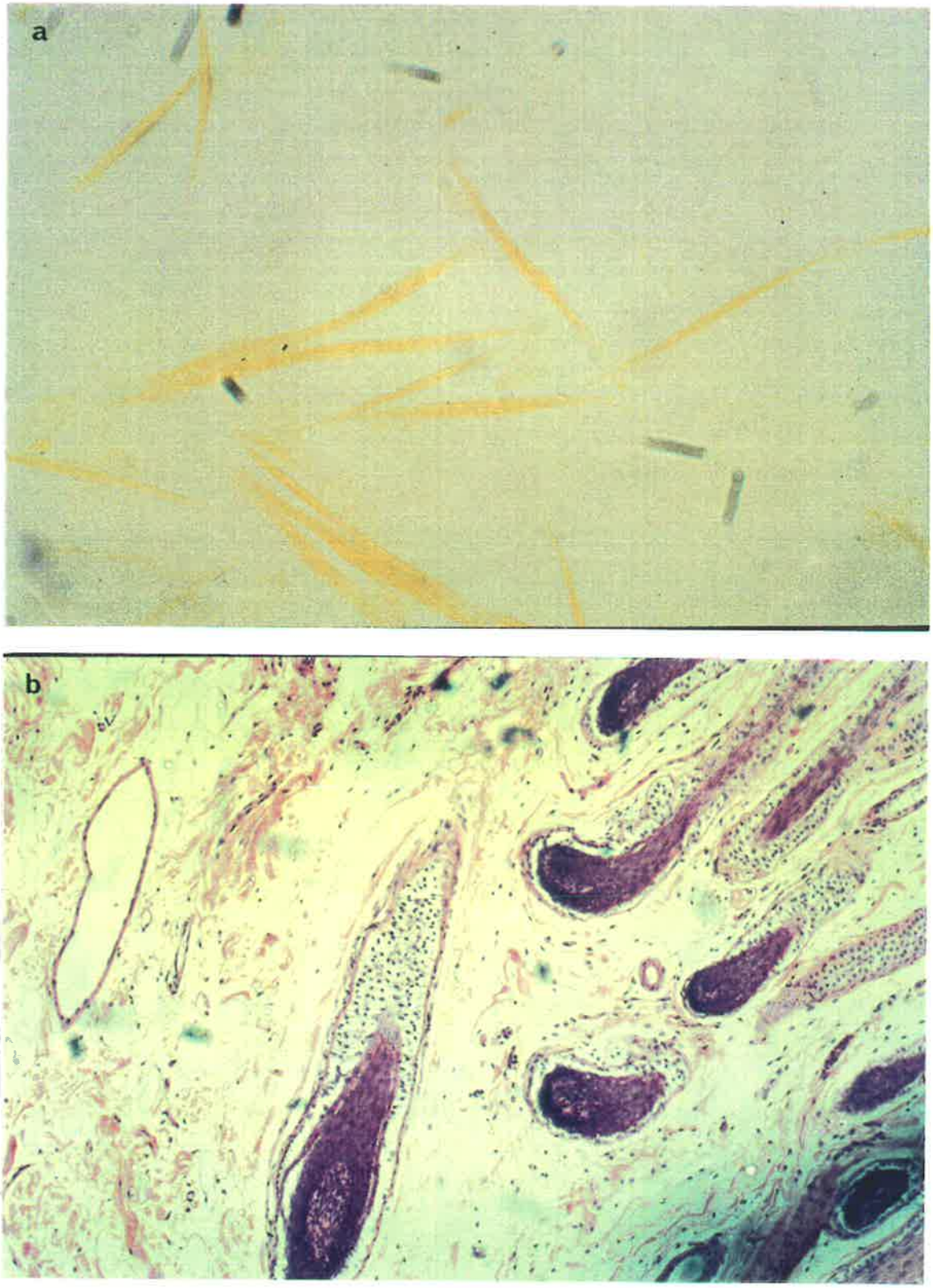

Plate 2.5. Photomicrographs of biological variation within individual sheep.

a) Variation of individual cortical cells from a strongwool Merino. The isolated cells were stained in picric acid. Magnification: 1188x.

b) Variation of bulb size within sheep from the control flock from the ANAMA stud. Note the large follicle on the left of the photomicrograph. Magnification 425x.

The number of cortical cells produced per hour differed significantly between strains ($P < 0.001$; Table 2.8) and the estimated proportion of bulb cells entering the fibre tended to be greater in the strongwool Merinos. The lack of statistical significance in the efficiency of fibre production between strains was due to the large variation between sheep, particularly in the strongwool Merinos where the proportion of bulb cells entering the fibre ranged from 12% to 56% (Appendix 3.1.1.). Cortical cell volume was correlated with both wool production per unit area and fibre production per follicle and the number of cortical cells produced per hour also was highly correlated with wool production per unit area of skin (Table 2.7.).

Table 2.8. Cortical cell length, width and estimated volume, the rate of cortical cell production and the ratio of this to the number of cells produced in the bulb (efficiency) of the finewool and strongwool Merinos (Means with s.e.m. in parentheses)

	Finewool	Strongwool	*P* =
Cortical cell volume (μm^3)	896 (28.0)	1061 (71.8)	0.059
width (μm)	5.8 (0.06)	6.5 (0.23)	0.010
length (μm)	93.2 (2.27)	88.6 (1.18)	0.099
Cortical cell production (h^{-1})	3.4 (0.32)	10.2 (0.85)	0.001
Efficiency	0.20 (0.028)	0.35 (0.077)	0.104

2.3.5. Determinants of fibre production per follicle

In the analysis of characteristics associated with fibre production per follicle, i.e. the number and volume of bulb cells and the rate at which they divided and the volume of individual cortical cells, it was not possible to include efficiency of fibre production or number of cortical cells since both of these were estimated directly from fibre volume. It was found that the number of cells per bulb and the volume of individual bulb cells accounted for 70% of the variance. The inclusion of genotype significantly improved the regression and the resulting estimation accounted for 91% of the variance in the volume of fibre cortex produced between sheep. Two distinct equations ($R^2 = 0.91$ for both)were required to describe fibre growth from the follicle (FV) as follows:

Finewool Merinos (Equation 2.13a.)

$$FV = 0.93(\pm 1.34) + 0.00136(\pm 0.00189)\ CN + 0.00788(\pm 0.00768)\ CV$$

Strongwool Merinos (Equation 2.13b.)

$$FV = 7.35(\pm 3.42) + 0.00136(\pm 0.00189)\ CN + 0.00788(\pm 0.00768)\ CV$$

where CN is number of cells per bulb,
CV is the volume of individual bulb cells, and
the values in parentheses are the standard errors of the term.

2.3.6. Determinants of wool production per unit area of skin

In an analysis of all of the measured characteristics, follicle density, bulb volume, the number of cells per bulb, the number of bulb cells produced per hour and cortical cell volume were modelled first and it was found that these accounted for 82% of the variance in wool production per unit area of skin. After removal of the least significant t-values, it was found that density and bulb volume accounted for 82% of the variance. Up to 97% of the variance in wool production per unit area of skin could be accounted for by the inclusion of a constant term for genotype. The inclusion of the other measured characteristics in the model, i.e. volume of individual bulb cells, bulb turnover time, germinative volume, number of cortical cells produced per hour and the proportion of bulb cells entering the fibre, did not significantly improve the correlation ($R^2 = 0.98$). Thus the equation which best predicted wool production per unit area of skin (WG) for the finewool Merinos was

(Equation 2.14a.)

$$WG = -0.68(\pm 0.103) + 0.0172(\pm 0.0033)\ D + 3.5 \times 10^6(\pm 1.04 \times 10^6)\ BV$$

$$(R^2 = 0.97)$$

and for the strongwool Merinos, the equation was

(Equation 2.14b.)

$$WG = -0.02(\pm 0.288) + 0.0172(\pm 0.0033)\ D + 3.5 \times 10^6(\pm 1.04 \times 10^6)\ BV$$

$$(R^2 = 0.97)$$

where D is follicle density,
BV is the volume of the follicle bulb, and
the values in parentheses are the standard errors of the term.

EXPERIMENT 2.

2.3.7. Wool growth, liveweight and feed intake

The sheep from the stud flock at ANAMA Merino stud produced 40% more wool per unit area of skin than those from the control flock and this difference was significant (P = 0.047; Table 2.9.). There was no difference between the two flocks in the average volume of fibre cortex produced per individual follicle. This was due to the similar fibre diameters and length growth rates of the two flocks over the last five days of the trial.

Table 2.9. Wool production per unit area of skin (wool), fibre diameter, length growth rate and the volume of fibre cortex produced per follicle (fibre cortex) of control and stud sheep from the ANAMA stud (Means with s.e.m. in parentheses)

	Control*	Stud*	*P* =
Wool (mg cm^{-2} day^{-1})	0.95 (0.087)	1.20 (0.078)	0.047
Fibre diameter (μm)	24.0 (0.82)	24.7 (0.41)	nsd†
Fibre length growth rate (μm day^{-1})	355 (15.8)	379 (9.6)	nsd†
Fibre cortex (μm^3 x 10^3 h^{-1})	7.5 (0.75)	8.1 (0.30)	nsd†

* Wool weights were measured over the last 20 days of the trial period and other measurements were made over the last five days of the trial

† No significant difference

All sheep gained weight during the 98 day stabilisation period with animals in the stud flock showing the greatest increase in liveweights. There was a significant difference between the two flocks in both the initial and final liveweights (P < 0.001; Table 2.10.)

Feed was allocated to the animals at a rate of 30g of pellets per kilogram liveweight during the first 67 days of the trial, was reduced to 25g per kilogram liveweight for 39 days and ultimately reduced to 22g per kilogram liveweight for the final 23 days. This was done since there was a high degree of variation in feed intake per kilogram liveweight between sheep (Appendix 3.2.2a,b.). In the period during which wool production per unit area of skin and individual follicle production rates were determined, the majority of sheep consumed at least 20g per kilogram liveweight with the exception of one control sheep and two stud sheep. During the 20 days of the stabilisation period, feed intake per kilogram liveweight did

not differ between the two flocks, thus achieving the objective of differences in wool production rates under similar nutritional conditions. In fact, wool production per unit area of skin and the volume of fibre cortex produced per follicle were not correlated with feed intake per unit liveweight (r = 0.15 and r = 0.08, respectively), nor with total intake per day (r = 0.39 and r = 0.18, respectively).

Table 2.10. Liveweights at the commencement and conclusion of the trial, feed intake during the final wool measurement periods and efficiency of wool production per unit feed intake of the control and stud flocks from the ANAMA stud (Means with s.e.m. in parentheses)

	Control	Stud	*P*<
Initial liveweight (kg)	47 (1.4)	60 (1.4)	0.001
Final liveweight (kg)	54 (2.1)	75 (2.0)	0.001
Feed intake during final 20 days (g/kg/d)	21 (0.2)	21 (0.4)	nsd†
Feed intake during final 5 days (g/kg/d)	21 (0.4)	21 (0.5)	nsd†
Efficiency of wool growth (mg/kg)	0.82 (0.070)	0.78 (0.060)	nsd†

† No significant difference

2.3.8. Skin and follicle characteristics

The stud sheep had significantly more follicles per area of skin than the sheep from the control flock (Table 2.11.). Surprisingly, follicle density was not significantly correlated with wool production per unit area of skin when the sheep were treated as one group (Table 2.12.), however the follicle density of the sheep in the stud flock tended to be positively correlated with wool production per unit area of skin (Table 2.12.; $P < 0.1$). There was no correlation in the control flock (Table 2.12.). There was also no correlation between follicle density and the volume of fibre cortex produced in either of the two groups of sheep.

Table 2.11. Follicle density, volume of the germinative tissue in the bulb (bulb volume), and total germinative volume of the skin (total germ. volume) for control and stud sheep from ANAMA stud (Means with s.e.m. in parentheses)

	Control	Stud	*P* =
Follicle density (mm^{-2})	44.8 (3.03)	56.9 (4.43)	0.034
Bulb volume ($\mu m^3 \times 10^4$)	15.5 (0.76)	15.7 (0.61)	0.547
Total germ. volume ($\mu m^3 \times 10^5\ mm^{-2}$)	66.0 (3.30)	88.1 (4.7)	0.002

The volume of germinative tissue in the bulb was similar between the two flocks (Table 2.11.), however within each sheep there was a large variation between the volume of individual bulbs (Appendix 3.2.1.). Plate 2.5b. shows this difference in bulb size within a single sheep with two bulbs adjacent to one another. This variation was evident in both flocks with the standard deviation ranging from 33% to 51% and 22% to 47% of the mean of volume of germinative tissue in 25 bulbs in the control and stud flocks, respectively.

Table 2.12. Relationships between follicle and skin characteristics and both wool production per unit area (Wool production) and the production of fibre cortex per follicle (Fibre volume) (Correlation coefficients)

Wool production	Combined	Control	Stud
Density	0.37	-0.21	0.57
Bulb volume	0.18	0.55	-0.42
Germinative volume	0.51 *	0.39	0.38
Fibre volume			
Bulb volume	0.47 *	0.66 *	-0.11

* $P < 0.02$

In the control sheep, there was a significant positive correlation between the average volume of germinative tissue in the bulbs and fibre production per follicle (Table 2.12.; $P < 0.02$) and there tended to be a positive correlation with wool production per unit area of skin ($P < 0.1$). There was no significant correlation in the stud sheep between the volume of germinative tissue in the bulb and either wool growth per unit area of skin or fibre cortex production per follicle (Table 2.12.). When the two flocks were combined, there was a significant correlation between the volume of germinative tissue in the bulb and the volume of fibre cortex produced ($P < 0.05$) but not with wool production per unit area of skin (Table 2.12.).

Follicle density was negatively correlated with the volume of germinative tissue in the bulb (Fig. 2.5. $r = -0.46$; $P < 0.05$) when the two flocks were combined. The total volume of germinative tissue in the skin was not correlated with either of the wool production estimates in the individual flocks but was significantly correlated with wool production per unit area of skin when the two flocks were combined (Fig. 2.6.; Table 2.12.).

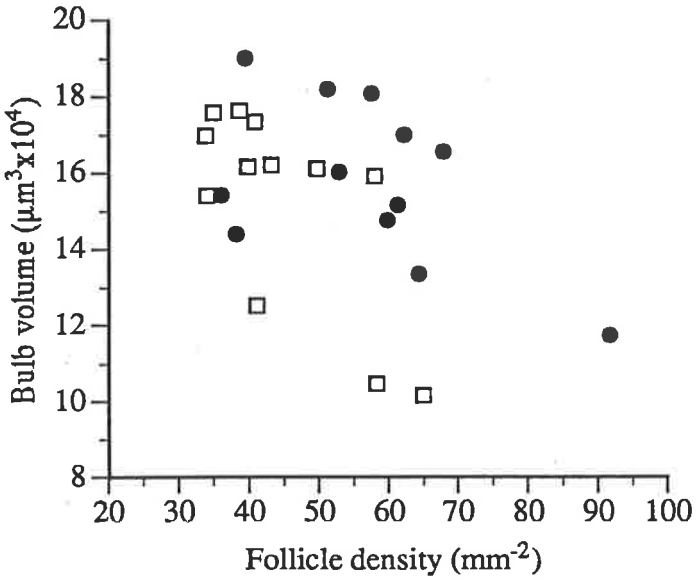

Figure 2.5. The relationship between follicle density and the volume of germinative tissue in the follicle bulbs of sheep from the control (□) and stud flocks (●) from ANAMA. (r = -0.46; P < 0.05)

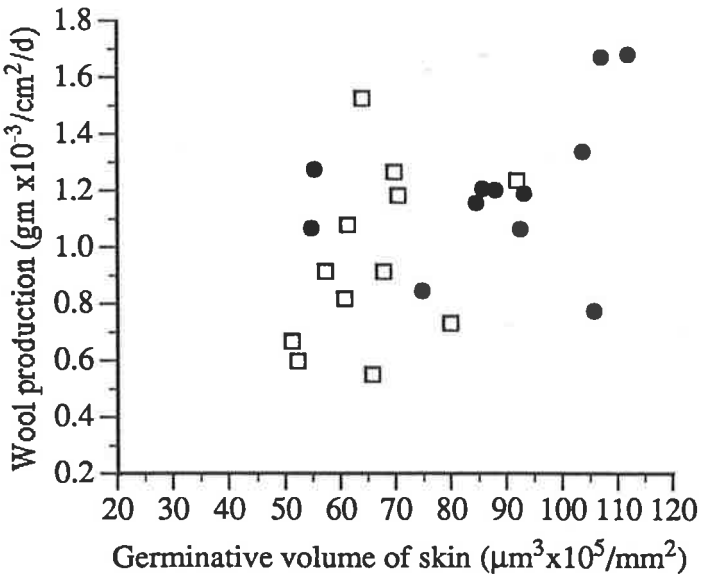

Figure 2.6. The relationship between the total volume of germinative tissue in the skin and wool production per unit area of skin of sheep from the control (□) and stud flocks (●) from ANAMA. (r = 0.51 ; P < 0.05)

2.3.9. Determinants of wool production

The general equations obtained in experiment 1, $WG = \mu + \beta_1 D + \beta_2 BV$ (Eq. 2.14) and $FV = \mu + \beta_3 BV$ (Eq. 2.13), were fitted to the data from experiment 2. These equations only accounted for 20% of the variance in wool production per unit area of skin and 16% of the variance in the rate of production of fibre cortex. It was not possible to adequately describe either wool growth per unit area of skin, nor individual fibre production with these equations, which made it inappropriate to compare the equations generated from the two experiments. The distinct equations required for each genotype had different β_2 and β_3 coefficients as follows:

Control flock (Equation 2.15a)

$$WG = 1.03(\pm 0.808) + 0.0089(\pm 0.00498)D + \underline{2.1 \times 10^6}(\pm 4.01 \times 10^6)BV;$$

Stud flock (Equation 2.15b)

$$WG = -0.77(\pm 0.808) + 0.0089(\pm 0.00498)D + \underline{8.7 \times 10^6}(\pm 4.57 \times 10^6)BV;$$

Control flock (Equation 2.16a)

$$FV = -2.3(\pm 4.70) + \underline{6.5 \times 10^5}(\pm 3.00 \times 10^5)BV; \text{ and}$$

Stud flock (Equation 2.16b)

$$FV = 9.0(\pm 3.72) - \underline{0.6 \times 10^5}(\pm 2.34 \times 10^5)BV.$$

These equations accounted for 35% of the variance in wool production per unit area of skin and 30% of the variance in individual fibre production.

2.4. DISCUSSION

Experiment 1 reported in this chapter is one of the most detailed studies undertaken of the relationships between wool growth and follicle characteristics of two groups of sheep maintained under similar environmental conditions. Investigations to determine possible causes of genetic differences in wool growth have used either selection lines, such as the Trangie fleece weight flocks (Dun, 1958; Williams, 1966; Williams & Winston, 1987) or, as in the present work, strains of Merinos (Weston, 1959; Dunlop *et al.*, 1966; Saville & Robards, 1972). Thus, both selection flocks and strains of Merinos have been utilised to

provide sheep sufficiently different to enable the detection of factors associated with these differences in wool growth, whilst conserving the basic characteristics of the Merino. In the present work a 2.5-fold difference in the rate of wool growth per unit area of skin and a 3.5-fold difference in volume of fibre produced per follicle existed between the two strains (Table 2.1). Thus, any major difference in follicle population and functioning between the two groups of sheep should be easily identifiable.

It is recognised that any difference or correlation observed in Experiment 1 can not be entirely attributed to genetic differences in so far that maternal and pre-experimental environments of the sheep were dissimilar. Nevertheless, the stabilisation period was long enough (91 days) to remove any transient environmental effects (Hynd 1982).

Wool growth rates differed significantly between the two strains despite there being no difference in feed intake per unit liveweight (Section 2.3.1). These results support the conclusions of other workers, that sheep with high rates of wool growth are more efficient converters of feed to wool (Weston, 1959; Dunlop *et al*, 1960; Schinckel, 1960; Dolling & Moore, 1960, 1961; Ahmed *et al.*, 1963; Williams & Winston, 1965; Dunlop *et al.*, 1966; Williams, 1966; Wodzicka-Tomaszewska, 1966; Dolling & Piper, 1968; Langlands & Hamilton, 1969; Saville & Robards, 1972; Joyce *et al*, 1976; Sumner, 1979), with the strongwool Merinos in the present experiment being 49% more efficient than the finewool Merinos (Table 2.2). This difference was not significant due to the large variation in intake between sheep-within strains and thus in the efficiency of wool production (Appendix 3.1.1.a. &b.). The results of the present experiment are interpreted on the assumption that the genetic capacity of wool production is independent of the total quantities of nutrients available to the sheep, as proposed in Section 1.2.2. Thus, the difference in wool growth rates between the two strains was not due to differences in feed intake per unit liveweight, but rather a result of inherent differences between the two groups.

2.4.1. Individual fibre production.

The most important aspect of the results of Experiment 1 is that all the measured morphological features and rate processes were greater in the strongwool than the finewool Merinos. It is concluded that no single, functionally-related character is responsible for the genetic control of wool production, rather it is a cumulative effect of a number of

characteristics. Strongwool Merinos produce more wool on a per follicle basis than finewool Merinos due to a combined effect of a high rate of bulb cell production, more of these cells entering the fibre and a larger number and volume of individual cortical cells in the fibre.

The strongwool Merinos had 116% more germinative tissue in individual bulbs than the finewool Merinos. This is in agreement with Williams and Winston (1987) who found a significantly greater area of mitotically-active tissue in the Fleece-plus flock than in the Fleece-minus flock. This finding was further supported by Experiment 2. There was no difference between the stud and control flocks in the volume of cortex produced, and likewise no difference in the volume of germinative tissue in the bulb. Nevertheless, the two factors were correlated when the flocks were combined (Table 2.12).

In Experiment 1, differences in the volume of germinative tissue in the bulb was due to the presence of more and larger cells in the bulb of the strongwool Merinos. A regression analysis of characteristics directly associated with individual fibre production (Equations 2.13a & b) revealed that the number and volume of the cells in the bulb and genotype accounted for 91% of the variance of fibre production within and between strains. This relationship supports the conclusion that, for between sheep variation, the average volume of bulb germinative tissue is important in determining the average volume of individual fibres.

It is proposed that the volume of tissue in the bulb which is capable of cellular proliferation appears to be determined by genotype. This genetic control may be associated with the founder cell theory proposed by Moore *et al.* (1989) who hypothesised that the number of dermal papilla cells involved in follicle initiation determines fibre size. This genetically-controlled population of papilla cells may therefore determine the volume of tissue capable of proliferation in the bulb, which, in turn, is associated with fibre production per follicle.

The rate of cell production in the follicle bulb of the strongwool Merinos was almost twice that of the finewool Merinos (Table 2.6). These rates were comparable to those reported by others (Schinckel 1962; Short *et al.* 1965; Wilson & Short 1979; Hynd 1989a), although the range of cell production rates within the strongwool Merinos was greater than that reported previously (Hynd 1989a). This may be a consequence of an increased sensitivity to cell kinetics of the BrdU technique than the traditional colchicine method. In

fact, Table 2.5 indicates that there is more than a two-fold difference in the rates of cell division between the two techniques. These estimates are similar to those of Hynd and Everett (1990) who concluded that colchicine depresses cell birth rate but the possibility that BrdU overestimates cell birth rate cannot be disregarded.

Recently, Holle and Harris (1992) examined the clearance rate of BrdU from the skin following an intracutaneous injection of BrdU. They concluded that the cutaneous dose acted as a constant infusion, since free BrdU was detectable in the skin at least 24 hours after injection. It has been reported that 26% of the BrdU present in arterial blood 30 minutes after intraperitoneal injection was still present in the blood 6 hours after the dose in rats (DeFazio *et al.*, 1987). No reports were found pertaining to the clearance of BrdU from the circulation after intravenous infusion, thus for the present study, it was assumed that free BrdU would be removed from the circulatory system four hours after the dose was administered. If this did not occur, then the increase in the number of BrdU-labelled cells between four and eight hours after the infusion may not be solely due to cell division. Rather it may be due to a leakage of BrdU back into the cells, thus it is recognised that the BrdU may have provided an overestimate of cell division rate in Experiment 1.

BrdU affects differentiation of cells *in vivo* (Miura & Wilt, 1971; Adelson *et al.*, 1991) although there is no effect on cell proliferation or on the cell cycle (Miura & Wilt, 1971). The compound was suitable for the present study of bulb cell kinetics since wool production estimates were made prior to BrdU administration and any effect BrdU has on differentiation would not have distorted the present results. The decision to use BrdU to estimate cell division rates in the present study was due to the discontent expressed by others with the traditional stathmokinetic technique (Williams & Winston, 1987; Hynd, 1989a) and the observation that there was an apparent inhibition or lag time in the finewool Merinos when colchicine was administered in Experiment 1. This 'lag' was further investigated by studying the number of cells accumulated in metaphase prior to the initial sample taken one hour after colchicine injection. It was found that there was a period before the linear accumulation of cells in metaphase occurred. Normal linear accumulation appeared to occur after the longer lag. This result can be extended to the finewool Merinos which may have a longer lag period, i.e. greater than one hour, than the strongwool Merinos due to a slower cell division rate.

This assumes that the lag is related to cell division. If the lag simply represents diffusion into the bulb cells, one would expect that the finewool Merinos would have a shorter lag time since they have a smaller bulb volume than the strongwool Merinos. This lag time is not unusual *in vivo*, having been reported in other mammalian species (Al-Dewachi *et al.*, 1975a, b; Nome, 1975; Appleton, 1977). It was decided that the immunohistochemical technique would be the most appropriate method to estimate follicle cell kinetics in the present study since BrdU was likely to give a more accurate estimation of cell division rate, in particular in the finewool Merinos. It would still be valid to use the colchicine estimates since there was linear accumulation of metaphases after the lag phase, but as there was a difference in the length of this period it was decided that an underestimate of the rate of cell division would be obtained in the finewool Merinos (reviewed by Wright & Appleton, 1980).

There was no significant difference between the two strains of Merinos in the bulb turnover time, i.e. the time taken for the cell population of the germinative region of the bulb to renew itself (Fraser, 1965). However, the finewool Merinos did have a 33% slower turnover time than the strongwool Merinos. This lack of difference between the two strains could be attributed to the fact that although the strongwool Merinos had a higher rate of cell division, there was a larger volume of tissue in the bulb to replace. This may correlate with Williams and Winston's (1987) finding of no difference between the Fleece-plus and Fleece-minus flocks in mitotic density, i.e. the number of mitotic nuclei per unit volume of tissue. Fraser (1965) stated that '...as mitotic density is a number referring to a unit volume, it is no index of mitotic activity or mitotic rate...'. If Williams and Winston (1987) had not expressed mitotic rate per unit volume an alternative conclusion may have been made. They reported a difference in the area of mitotically - active tissue which suggests that the mitotic activity of the follicles differed between genotypes in order to be no difference in mitotic density. This is fundamentally equivalent to the present turnover time result which is an expression relating cell division rate and cell number. Both cell division rate and cell number differed between the finewool and strongwool Merinos and this ultimately resulted in no difference in turnover time between the two strains.

One of the assumptions inherent in the estimation of bulb turnover time is that all cells in the 'germinative' region of the bulb are capable of division. There have been reports that

bulbs exhibit asymmetrical mitotic activity associated with bilaterally - segmented fibres (Fraser, 1964; Adelson *et al.*, 1991). Observations in the present experiment did not indicate any asymmetry in cells labelled with BrdU in either strain (Plate 2.4), nor was there any asymmetry in the accumulation of metaphases when the colchicine technique was used (Plate 2.3), in agreement with observations by others (Williams & Winston, 1987; Hynd, 1989a). Furthermore, long-term labelling studies with BrdU indicate that the growth fraction (i.e. the proportion of bulb cells capable of division) in wool follicle bulbs is 1.0 (Hynd, unpublished). The turnover time estimates in the present study, therefore, were not biased by the follicle populations having a varied distribution of germinative tissue.

Cortical cell volume was estimated using an alternative method to that proposed by Short *et al.*, (1965). Preliminary studies (Appendix 2) indicated that fibre dispersion by HCl distorted the cells more than formic acid pretreatment, whereas trypsin digestion failed to release adequate numbers of cells to provide a representative sample of the fibre. The mean cortical cell volume did not differ between strains, in agreement with Williams and Winston (1987). In the present experiment strongwool Merinos tended to have a greater average cell volume than the finewools ($P = 0.055$), but within sheep variation was high (Plate 2.5a). This probably reflects differences in cortical cell proportion (Orwin *et al.*, 1984). Cortical cell volume was correlated with fibre production per follicle and this association may also be due to cortical cell type, since it is known that fibres with large diameters tend to have a high proportion of large orthocortical cells (Orwin, 1989). Discrete populations of cortical cell type could not be separated on the basis of cell size alone, since there was no clear bimodal distribution of the cortical cell parameters in the present study. Attempts were made to differentiate cell type by differential staining with methylene blue (Clarke & Maddocks, 1965), however this was unsuccessful (Appendix 2).

The number of cortical cells produced per fibre differed significantly between strains, in agreement with Williams and Winston (1987). This is not unexpected since cortical cell production is estimated directly from the volume of cortex produced per follicle (Equation 2.10) which were highly significantly different. New techniques need to be developed to obtain an independent estimate. An attempt was made to completely disperse cortical cells from a known volume of wool and then count the cells but it was impossible to adequately disperse all of the fibre without breaking individual cells (Appendix 2).

The present results support, but do not add, to the hypothesis that the distribution of bulb cells to the fibre and inner root sheath is under genetic control, as proposed by Short *et al.* (1965), Wilson and Short (1979), Hynd (1989a,b) and Hynd and Scobie (unpublished; cited by Hynd, 1989b). In the strongwool Merino there were 75% more cells entering the fibre than in the follicles of the finewool Merinos, although the difference between strains was not statistically significant due to a large within-strain variation (Table 2.8). It was not possible to regress this efficiency of fibre production against volume of fibre cortex, since efficiency is derived directly from fibre cortex production (Equation 2.11.). In order to further understand and accurately describe the constraints to the efficiency of fibre production, it is necessary a) to gain an understanding of the role of the inner root sheath and b) to develop methods which either determine efficiency of fibre production directly, or estimate the number of cortical cells produced per hour without directly using fibre volume. Alternatively, it may be possible to use the 'production ratio', i.e. the ratio of the cross-sectional area of the fibre to the cross-sectional area of fibre plus IRS, to provide an indication of the distribution of cells between the fibre and inner root sheath (Butler and Wilkinson, 1979; section 1.5.3) and it has been shown that there is a correlation between production ratio and cell distribution ($r = 0.63$, $P < 0.01$, $n = 18$; Hynd, unpublished)

2.4.2. Determinants of wool production per unit area of skin

Both follicle density and bulb volume have been found to be associated with wool production (Henderson, 1965; Brown & Turner, 1968; Barlow, 1974; Williams, 1987) but have never been studied concurrently. The stepwise regression analysis indicated that follicle density and the mean volume of germinative tissue in the bulb best predicted wool production per unit area of skin (Equation 2.14a, b) in Experiment 1. Similarly, the total germinative volume of the skin, i.e. the product of density and bulb volume, was found to be highly correlated with wool production per unit area of skin ($r = 0.91$; Fig. 2.3). This association of density and germinative volume of the bulb with wool production per unit area was examined further in a flock selected using a WOOLPLAN index and a randomly-bred flock to determine if the relationship is similar in commercial flocks (Experiment 2). It was confirmed that total germinative volume of the skin was significantly correlated with wool

production per unit area of skin ($r = 0.52$; $P < 0.05$), suggesting that these two characteristics are important components in determining inherent levels of wool production.

Total germinative volume of the skin accounted for 83% of the variance in Experiment 1 in contrast to only 26% of the variation in wool production in Experiment 2. This difference between the experiments was not unexpected since the relationship of various measured characteristics between the two groups of sheep within each experiment was quite disparate. In particular, the low-wool producers (finewool Merinos) of Experiment 1 had a higher follicle density than the high-wool producers (strongwool Merinos), but the reverse occurred in Experiment 2. In fact, when the multiple linear equations generated in Experiment 1 were fitted to the data of Experiment 2, the equations did not adequately describe the results, suggesting that these equations are not suitable for generally estimating wool production per unit area of skin. It is unlikely that a general equation could be generated for the determinants of wool production for all sheep, rather an expression would be required for each group of sheep for every set of conditions. With an adequate, basic understanding of the factors controlling wool production, future computer simulations may be able to predict wool growth rates more accurately than is presently possible.

4.4.3. Conclusions

The results presented in this chapter support the initial hypothesis that differences in wool production between sheep under similar environmental conditions are due to differences in the follicle population of the sheep. Wool production levels were associated with the total volume of germinative tissue in the skin, i.e. the number of follicles and the volume of germinative tissue in the follicle bulbs. The present results suggest that the relative importance of the two factors; i.e. follicle density and volume of germinative tissue in the bulb, is dependant on individual conditions associated with each group of sheep. For example, the high wool-producing strain of strongwool Merino had a lower follicle density and a greater volume of germinative tissue in individual bulbs than the lower wool-producing strain of the Camden Park Merino. In contrast, the high wool-producing stud flock from Experiment 2 had a higher follicle density and an equivalent average volume of germinative tissue in the bulb compared to the randomly-bred flock. Nevertheless, in both experiments the high wool-producing sheep had a greater total volume of germinative tissue in the skin

than the low wool-producing sheep. It is concluded that there is a difference in the **follicle population**, i.e. the relative importance of follicle density and volume of germinative tissue in the bulb, between sheep with inherent differences in wool production.

The second part of the hypothesis, that differences in wool production in sheep kept under similar environmental conditions are due to differences in the functioning of individual follicles, was not supported. The results of Experiment 1 indicated that there was no major functional difference between the follicles, rather wool production levels were due to a cumulative effect of small differences in a number of characteristics. If the functioning of individual follicles did differ, one may have expected a similarity in most of the characteristics and a large difference in one of the characters, for example a different distribution of bulb cells between the fibre and the inner root sheath. It is concluded;
a) that the follicles of the high wool-producing sheep were capable of producing more wool due to an additive effect of individual morphological features and rate processes which were greater than those in the low wool-producing sheep, and
b) that no single, functionally-related character is responsible for the genetic control of wool production.

Given that the functioning of individual follicles is similar throughout the Merino population, the mechanisms by which the high level of wool production of the South Australian strongwool Merino is maintained remains to be determined. There must be some mechanism which enables follicles that have similar modes of action to be capable of producing wool at higher rates than follicles on other sheep. This may lie in the nutrient supply to the follicle and the subsequent ability of the follicle to utilise nutrients essential for wool production. These will be examined in more detail in the following chapters.

Chapter 3.

Skin blood flow of finewool and strongwool Merinos

CHAPTER 3. SKIN BLOOD FLOW OF FINEWOOL AND STRONGWOOL MERINOS

3.1. INTRODUCTION

There are no published reports comparing blood flow to the skin of different strains of Merinos nor the relationship between wool growth and skin blood flow. The cutaneous circulation of sheep has been measured under various conditions, such as during heat stress and exercise, and it is believed that there is a relationship between skin blood flow and wool production (Section 1.2.5). Black and Reis (1979) developed a simple 3-pool simulation model to predict the utilisation of amino acids in sheep. They showed that there could be up to a 70% increase in wool growth when blood flow to the skin is doubled, although the response to increased blood flow was shown to increase at a diminishing rate.

Many methods exist for the study of cutaneous circulation and its morphology. Three different physiological parameters are used when describing skin blood flow - physical movement, heat transport and oxygen content (reviewed by Swain & Grant, 1989). Both methods used in the current study of skin blood flow, i.e. radiolabelled microspheres and laser Doppler velocimetry, utilise the physical movement parameter. These are the only techniques which will be discussed.

Radiolabelled microspheres have been used extensively in sheep for studying blood flow distribution and thermal stress (Hales, 1973a, b; Hales *et al.*, 1976; Hales, *et al.*, 1978a,b; Bell, *et al.*, 1983) and the general technique and its use has been extensively reviewed by Heymann *et al.* (1977). Briefly, the microsphere technique is an indicator fractionation technique, based on the Fick principle that mass is conserved in the circulation and mass is derived from the product of volume and concentration (Sapirstein, 1958). When a known quantity of microspheres are injected into the left cardiac ventricle, the spheres mix and flow with the arterial blood whilst being distributed in the same manner as the blood. The microspheres become lodged in the vessels corresponding to the size of the sphere and the quantities of microspheres can subsequently be assessed by determining the radioactivity

present in the tissue of interest. This gives an estimate of the amount of blood flowing to the tissue.

The use of microspheres to measure tissue blood flow has advantages over other methods (e.g. isotope clearance technique). In particular, the number of spheres in a tissue sample is a function of blood flow, and the distribution of the microspheres is not affected by the permeability, surface area or metabolic activity of the vessels or surrounding tissues. The microsphere technique is often the standard against which other techniques are validated but there are inherent disadvantages with using the technique. Firstly, the method is invasive and acute since the animal undergoes surgery for catheter insertion and is slaughtered before tissue sampling. The use of microspheres also involve additions to and withdrawals from the circulatory system which possibly upset the haemodynamic parameters of the blood (Flaim *et al.*, 1978; Stanek *et al.*, 1985), the technique estimates blood flow to (not through) the tissue and, finally, there are shunting problems dependant on the architecture of the vessels (reviewed by Shepherd & Jacobsen, 1981). To overcome some of these problems, alternative techniques suitable for the estimation of skin blood flow have been developed.

Laser Doppler velocimetry (LDV) is a non-invasive, isotope-free technique which provides continuous measurement of blood flow through the skin. The use of a laser beam to measure blood flow was first described by Stern (1975), further developed by Holloway and Watkins (Holloway & Watkins, 1977; Watkins & Holloway, 1977) and improved by Nilsson *et al.*, (1980). By placement of a probe on the skin surface, laser light with an infra-red wavelength of 780nm is transmitted into the skin through a fibre optical cable and the reflected signal is returned through a second cable to the instrument. The light does not affect blood flow since wavelengths greater than 450nm are considered not to interact with red blood cells (Furchgott *et al.*, 1961). The light frequency reflected from stationary tissue is unchanged, however that reflected from moving red blood cells undergoes a frequency shift according to the Doppler equation - $f = \frac{2v f \cos\theta}{c}$

where v is the velocity of the red blood cells
f is the frequency of the incident laser light
θ is the angle between the incident light and the direction of motion of the erythrocytes
c is the speed of light.

The Doppler shift is converted by the electronics of the machine to give an output which is linearly proportional to red blood cell flux (number x velocity).

The laser Doppler velocimeter is used extensively in the assessment of microcirculation and generally there is good agreement between LDV and other methods. Examples include the isotope clearance technique (Engelhart & Kristensen, 1983; Engelhart *et al.*, 1988), plethysmography (Johnson *et al.*, 1984), video microscopy (Tyml & Ellis, 1985) and measurement with a blood flow meter (Pershing *et al.*, 1989). LDV is sensitive to movement artifacts and it was suggested that it is difficult to perform true quantitative measurements (Tenland, 1982; cited by Tenland *et al.*, 1983). Difficulties arise from variation in blood flow from comparable sites in the same individual and also from the same site in the same individual over time. This variation is possibly due to the arrangement of the underlying microvasculature (Braverman *et al.*, 1990). To overcome these limitations, multiple readings are taken at a number of sites within a defined area and the mean calculated.

The aim of this experiment was to develop and validate the use of a laser Doppler velocimeter for the measurement of blood flow in the skin of sheep. The hypothesis that differences in wool growth between finewool and strongwool Merinos are associated with differences in blood flow through the skin was then tested.

3.2. EXPERIMENTAL PROCEDURE

3.2.1. Design

The experiment reported in this chapter consisted of three individual trials (Experiments 3.1, 3.2 and 3.3) which involved strongwool and finewool Merino wethers aged 3 - 4 years. They were fed a daily ration of grain-based sheep pellets (1kg/d) and water was available *ad libitum*. All sheep were housed indoors in individual pens before being moved to a climate-control room (Light between 0830 and 1930) and housed in metabolism crates.

Wool growth estimates were made using the tattoo patch technique (Section 2.2.3.) during the 14 days immediately prior to the sheep being moved to the climate-control room. The design of each experiment is summarised in Table 3.1 and discussed individually (*vide-*

infra). Air temperature and relative humidity were recorded prior to the measurement of skin blood flow for each sheep using a maximum and minimum thermometer and a wet and dry bulb thermometer, respectively.

Table 3.1. Summary of experiments undertaken to validate and determine the optimal conditions for use of laser Doppler velocimetry (LDV) to measure blood flow through the skin of sheep

	No. Finewool	No. Strongwool	Measurements
Experiment 3.1	4	4	Day effect
			Region effect*
			Wool growth
Experiment 3.2	4	4	Position effect†
			Follicle density
			Wool growth
Experiment 3.3	2	4	Validation of LDV
(Original number§)	(5)	(7)	using microspheres

* Measurements were made within a small area of the midside

† Measurements were made on the midside and on the abdominal flank

§ The number of sheep originally in the experiment

3.2.1.1. Experiment 3.1

Four strongwool and four finewool Merinos were housed indoors for a 67 day stabilisation period during which wool growth estimates were made, before the sheep were transferred to the climate-control room. Blood flow was estimated using LDV as described below (Section 3.2.2.1.) at three sites along the dorsal edge of the midside tattooed patch. Each of these sites was measured twice on four consecutive days.

3.2.1.2. Experiment 3.2

Four strongwool and four finewool Merinos were housed indoors for a 46 day stabilisation period during which wool growth estimates were made before the sheep were transferred to the climate-control room. Blood flow was estimated using LDV as described in Section 3.2.2.1. at two positions on the right side of the sheep, i.e. within the tattooed midside patch (midside) and on the abdominal flank region (flank; Fig. 3.1.). Three measurements were made in each region on two consecutive days. Skin samples were taken

from both the midside and abdominal flank, processed, sectioned transverse to the follicle at 10μm and then stained with haematoxylin and eosin for the determination of follicle density as described in Section 2.2.4.1. Density was measured at a magnification of 337 times and estimated using Equation 2.2.

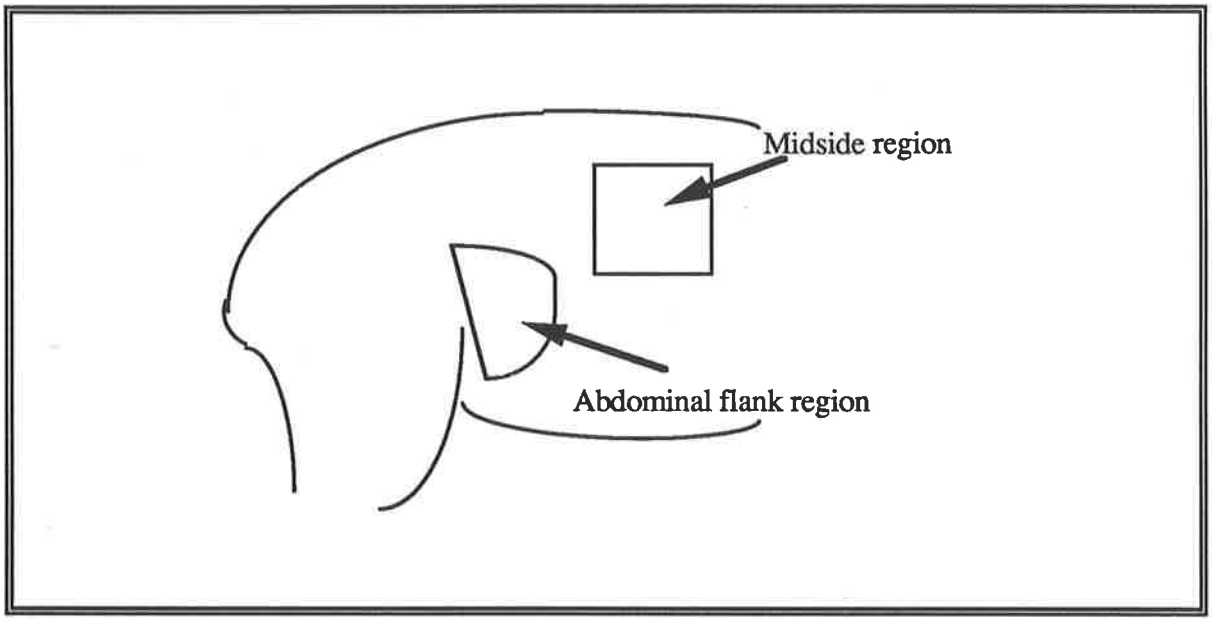

Figure 3.1. Midside and abdominal flank regions of the sheep where blood flow measurements were made with laser Doppler velocimetry in Experiment 3.2.

3.2.1.3. Experiment 3.3

Five finewool and seven strongwool Merinos were housed indoors for at least 14 days before being transferred to a climate control room (Temperature = 18°C; Light between 0830 and 1930). Blood flow was measured with LDV at three sites along the upper edge of the midside tattooed patch on two consecutive days prior to surgery. The sheep underwent surgery (Section 3.2.2.2.) in preparation for the application of the microspheres as described in detail by Hales (1974) and briefly in Section 3.2.2.3. One to two days after surgery, blood flow was estimated at three sites along the dorsal edge of the midside tattooed patch with LDV (Section 3.2.2.1). Radiolabelled microspheres were injected into the sheep and then tissue and blood samples were collected and analysed to determine blood flow to the skin and kidneys, as described in Section 3.2.2.4.

3.2.2. Materials and Methods

3.2.2.1. Laser Doppler technique

A laser Doppler velocimeter (Laserflo™, Blood perfusion monitor (403A) TSI Inc. St Paul, USA) with a right angle skin probe was used to estimate blood flow. This particular monitor has a solid state laser diode with a wavelength of 780 ± 20nm. It was necessary to use adhesive tape (5cm x 12cm; Leukoplast ® Beiersdorf, Hamburg) topped with double-sided sticky-tape with three holes punched (9mm diameter) approximately 15mm apart to attatch the probe to the skin (Plate 3.1.) as the recommended adhesive did not adhere to the skin of the sheep. The tape was placed on the skin, which had been closely clipped of wool prior to the first day of blood flow measurements, and the probe placed on the caudal edge of the tape. The sheep were then allowed to settle before blood flow was recorded for approximately 45 seconds on a continuous feed chart (100mm/minute). This measurement was repeated twice, moving the probe in a cranial direction. After completion of each days' measurements, estimates of blood flow were made directly from the chart paper. The monitor was calibrated with radiolabelled microspheres in dog gastric mucosa by the manufacturer, hence the units are arbitrarily designated as ml/min/100g tissue.

Plate 3.1. Photograph of the laser Doppler velocimeter probe placed within the midside tattoo patch of a sheep. The probe is attatched to the skin with adhesive tape topped with double-sided sticky tape.

3.2.2.2. General aseptic surgery procedure

Sheep were fasted for 24 hours before anaesthesia was induced with pentobarbitone sodium (i.v. 10 - 15ml; Nembutal®; Boehringer Ingelheim, Artarmon, NSW) and maintained with halothane (3%; Halothane-M&B; May and Baker Australia Pty. Ltd, West Footscray, Vic.) in oxygen. An intramuscular injection of procaine benzylpenicillin and dihydrostreptomycin (2ml; Pen & Strp. Injection; Norbrook Laboratories, UK) was given as a routine prophylactic treatment after surgery. Catheters were made with single lumen, polyethelene or clear vinyl tubing (Adelab Scientific, Adelaide, SA) and appropriately gauged needles (Teremo Pty Ltd, Melbourne, Vic.), sterilised with benzalkonium (0.1%; Zephiran; Winthrop Labs., Ermington, NSW) and flushed with heparinised saline (10 i.u./ml; saline and heparin sodium [5000U/ml] both were products of Commonwealth Serum Laboratories, Melbourne, Vic.). Patency of the catheters was maintained by the continuous infusion of saline with an infusion pump (Minipuls 2; Gilson, Villiers, France).

3.2.2.3. Insertion of catheters for microsphere administration

Aseptic surgical procedures (Section 3.2.2.2) were used to place catheters in the left cardiac ventricle via the left carotid artery and in the left deep circumflex iliac artery. An incision was made above the artery of interest and the vessel isolated and ligated with silk on the cranial edge of the incision. A small cut was made in the artery and a polyethelene catheter (i.d. 1.00mm, o.d. 1.50mm) was placed in the carotid artery. The catheter was passed down the artery and positioned in the left ventricle. The correct placement in the heart was determined by pressure recording with a strain gauge transducer and chart recorder (ICT-2H, Gilson Duograph Model). A second catheter constructed from clear vinyl tubing (i.d. 0.80mm, o.d. 1.20mm) was placed in the saphenous artery and inserted to 20cm of the tip of the catheter.

3.2.2.4. Microsphere administration

Microspheres 15μm in diameter and labelled with ^{57}Co were supplied suspended in saline (0.9%) with polyoxyethylene 80 sorbitan monooleate (0.01%, Tween 80; NEN-TRAC Microspheres; DuPont, Boston, MA). This size of microsphere was chosen since 15μm microspheres are generally used as a measure of true capillary flow (reviewed by Hales, 1974). The 15μm microspheres also satisfy the criteria of being distributed similarly to red blood cells as well as there not being significant non-entrapment of the

spheres by the blood vessels (Heymann *et al.*, 1977). Approximately 2ml of the microsphere-containing solution was mixed with saline (7ml) and gently agitated by hand, as recommended by Naredi *et al.*, (1991). The dose, containing approximately 2.2 x 10^8 microspheres, was injected rapidly into the left cardiac ventricle and the catheter was flushed with saline immediately after the injection via a three-way tap. An 'artificial organ' sample was withdrawn simultaneously from the saphenous artery for 30 seconds, starting five seconds prior to the administration of the microspheres. A second blood sample was collected for a further 15 seconds, immediately after the first sample. Blood was collected directly into preweighed vacuum sample-tubes (Venoject; Terumo, Elkton, USA) containing sodium heparin (143 i.u.) and then centrifuged to precipitate the microspheres (10 min; 6000*g*; 4°C; MSE Mistral 4L). Immediately after the collection of the blood, saturated magnesium sulphate (20 - 25ml; $MgSO_4.H_2O$, Kilsyth, NSW) was injected into the heart via the carotid catheter until the sheep was dead. Skin from the sides of the deceased sheep was carefully removed, leaving the superficial blood vessels attached to the animal. Small samples from the cortex of both the left and right kidneys were taken to verify equal distribution of the microspheres throughout the body. The skin samples (~ 10 x 10cm; 5 per side) and kidney samples (~ 1g per kidney) were placed into preweighed plasma tubes and then the samples and aliquots were weighed and assayed for gamma activity (1282 Compugamma, LKB Wallac). Blood flow to the skin and kidney were estimated using Equation 5.1, derived from Hales (1974).

Equation 5.1.

$$\text{Blood flow} = \text{Sampling rate of arterial blood} * \frac{\text{Radioactivity of tissue}}{\text{Radioactivity of arterial blood}}$$

3.2.3. Statistical analysis

Analysis of variance statistics were performed with GENSTAT 5 (1988, Lawes Agricultural Trust, Rothamsted Experimental Station) and the means and standard errors of the means were generated within this program. Each measurement was treated independently and the analyses included estimates of missing data, when appropriate. The analysis of variance statistics were used to test the effects of strain, site and/or day on blood flow within Experiment 3.1 and 3.2.

Simple correlation coefficients were estimated with the Cricket Graph computer software package (version 1.3.1; Cricket Software, Philadelphia, USA). These were used to determine the relationships between blood flow, wool growth and density (Experiments 3.1 and 3.2) and to compare the results obtained from the two methods used for estimating blood flow (Experiments 3.3).

The mean and standard error of the mean were routinely calculated with data obtained from samples within sheep and then from sheep within strains. The Students t-test was used to compare wool growth and density between strains.

Results were considered significant when $P < 0.05$.

3.3. RESULTS

3.3.1. General

All sheep consumed the offered feed throughout each experiment. The temperature and relative humidity of the climate-control room varied significantly between days in Experiment 3.1 ($P < 0.001$ and $P = 0.001$ respectively) with a range in environmental conditions occurring on all days of Experiment 3.1 (Table 3.2.). The temperature and relative humidity at which blood flow was measured did not differ between strains ($P = 0.450$) and sites ($P = 0.613$) nor were there any significant interactions. Temperature and relative humidity were significantly correlated with blood flow ($r = 0.48$, $P < 0.01$; & $r = 0.37$, $P < 0.01$, respectively; $n = 192$). It was unnecessary to use either factor as a covariate since neither removed a significant proportion of variance from blood flow.

Table 3.2. Temperature (temp) and relative humidity (RH) in the climate-control room during Experiment 3.1. (means and standard errors of the differences of means [s.e.d.])

Day	1	2	3	4	s.e.d.
Experiment 3.- Temp (°C)	26	23	21	19	0.90
range	21 - 30	21 - 26	19 - 22	17 - 22	
- RH (%)	69	77	78	79	2.3
range	82 - 61	83 - 72	90 - 71	85 - 70	

Conditions remained constant during blood flow measurements in Experiment 3.2 (temperature = 18°C, relative humidity = 71%) and Experiment 3.3 (temperature = 21°C, relative humidity = 65%)

3.3.2. Blood flow variation within individual sheep (Experiments 3.1 & 3.2)

Blood flow through the skin was measured at least once at each of three sites in the midside patch on four consecutive days. Measurements were not made when the temperature reached 30°C on day 1 and 25°C on day 2, thus missing values were estimated in the analysis of the data. The rate of blood flowing through the skin at different positions varied within individual sheep with the coefficient of variation ranging from 12% to 26% on days 3 and 4 of Experiment 3.1 (Appendix 4.1.). There was a significant difference in the rate of blood flow between different days ($P < 0.001$) but not at different sites on the midside patch ($P = 0.876$; Table 3.3.)

Table 3.3. Blood flow through the skin (ml/min/100g tissue) on different days and different sites on the midside patch in Experiment 3.1 (means and standard errors of the differences of means [s.e.d.])

	Day / Site number					
	1	2	3	4	s.e.d.	*P* =
Day	4.7	3.8	3.2	3.2	0.33	< 0.001
Site	3.8	3.6	3.8	-	0.27	0.876

The temperature and relative humidity were constant when measurements were made of blood flow on the midside patch and on the abdominal flank, thus the three sites in each region were used as replicates in Experiment 3.2. There was greater variation in the rate of blood flowing through the skin of the abdominal flank region than the midside region within each sheep with the coefficient of variation (CV) ranging from 23% to 62% in the flank and 15% to 40% in the midside (Appendix 4.1). The ANOVA indicated that there was a consistently higher average blood flow rate through the flank region (4.2 ml/min/100g tissue) than the midside (3.3 ml/min/100g tissue; $P = 0.050$). There was no difference in the rate of blood flowing through the skin of the midside and the abdominal flank between days ($P = 0.201$) and blood flow through the skin of the midside was not correlated with blood flow through the skin of the abdominal flank region ($r = 0.38$; Fig. 3.2).

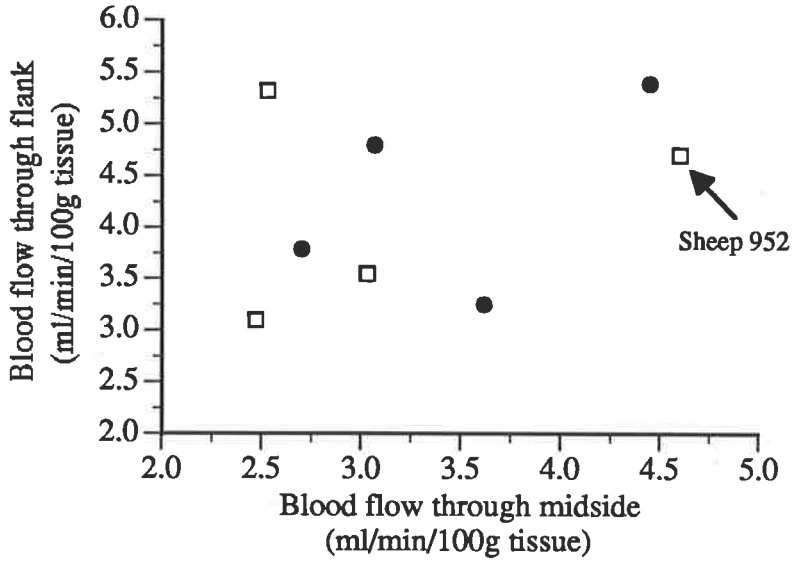

Figure 3.2. Relationship between blood flow through the midside and through the flank in finewool (□) and strongwool (●) Merinos [r = 0.38].

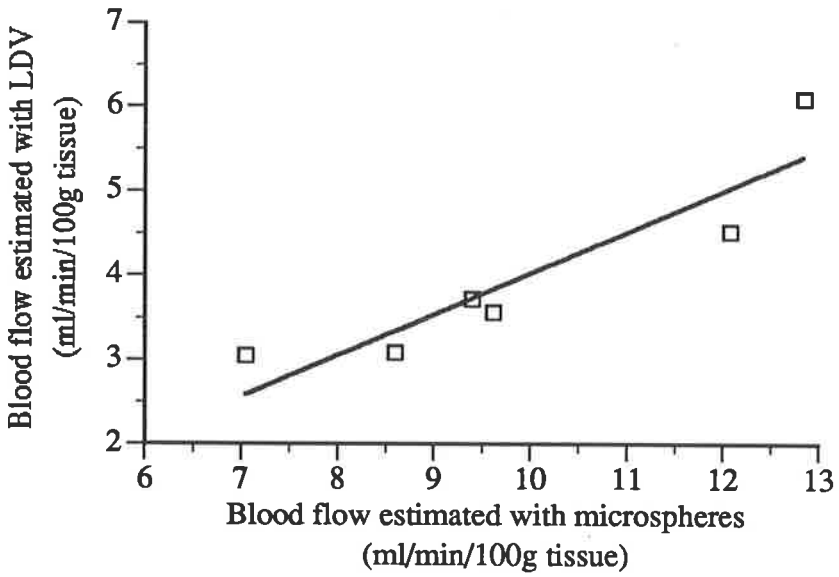

Figure 3.3. Relationship between blood flow through the midside measured using radiolabelled mirospheres and measured using the laser Doppler velocimeter (LDV) [r = 0.92; P < 0.02].

3.3.3. Validation of the laser Doppler velocimeter method (Experiment 3.3)

Three sheep died following surgery to insert catheters into the heart via the carotid artery and into the saphenous artery; two from suspected heart failure and the third from fluid in the lungs. The microsphere technique requires that the carotid cannula be situated in the left ventricle and that both the carotid and saphenous catheters remain patent. This occurred in two finewool and four strongwool Merinos and only these sheep were used for the validation of the LDV technique. Comparisons of the estimates of blood flow between the two strains were are not presented as it is difficult to obtain significant results with only the two finewool Merino survivors. The estimates of blood flow to the left and right kidney were significantly correlated ($r = 0.93$; $P < 0.02$) and differed by only between 3% and 17% (Table 3.4.). These results indicate that there was reasonable distribution of the microspheres around the body of the sheep, considering only one sample per kidney was collected. The temperature was 21°C and the relative humidity was 65% and both remained constant throughout the period in which the blood flow estimates were made.

Table 3.4. Blood flow (ml/min/100g tissue) to the left and right kidneys of the sheep used for the estimation of blood to the skin by radiolabelled microspheres

Sheep identification	Left kidney	Right kidney
987	711	731
971	1040	1072
978	603	557
988	871	747
989	1038	929
996	990	876

The mean of six blood flow estimates per sheep using LDV was 4±0.5 ml/min/100g tissue and ranged from 3 to 6 ml/min/100g tissue, whereas the estimates of blood flow obtained using microspheres had a mean of 10±1.0 ml/min/100g tissue and ranged from 7 to 13 ml/min/100g tissue. The absolute values of the two techniques differed significantly ($P < 0.001$) and the estimates obtained using the LDV were highly correlated with the radiolabelled microsphere estimates of blood flow to the skin ($r = 0.92$, $P < 0.02$; Figure 3.3.).

3.3.4. Blood flow in different strains of Merinos

There was a significant difference in blood flow through the skin between the finewool and strongwool Merinos in Experiment 3.1. (P = 0.007). Only the first three measurements on day 3 and 4 for each sheep of Experiment 3.1 were used for the following analyses in order to compare and combine Experiment 3.1 and 3.2. It was possible to combine these data since there was no difference in blood flow between days 3 and 4 in Experiment 3.1 (P = 0.913) despite a significant difference in temperature on these days (P = 0.045) but not relative humidity (P = 0.792).

In Experiment 3.1 there was a significant difference in the rate of blood flowing through the skin on the midside between the finewool and strongwool Merinos (P = 0.003; Table 3.5). In contrast, there was no difference between the two groups of sheep in Experiment 3.2 in either blood flow through the midside (P = 0.649) nor through the skin of the abdominal flank (P = 0.851; Table 3.5). The sheep in the latter experiment were not as intensively-trained to remain still as those in Experiment 3.1 so there was a lot more movement artifact in Experiment 3.2 due to feet stamping and restless movement backwards and forwards. The finewool Merinos appeared to be a lot more active than the strongwool Merinos, particularly 952 whose blood flow estimate is indicated in Figure 3.2.

Table 3.5. Blood flow through the skin (ml/min/100g tissue) on the midside patch and the abdominal flank (flank) in finewool and strongwool Merinos (means and standard errors of the differences of means [s.e.d.])

	Finewool	Strongwool	s.e.d.	*P* =
Experiment 3.1. - midside	2.8	3.6	0.19	0.003
Experiment 3.2. - midside	3.2	3.5	0.63	0.649
Experiment 3.2. - flank	4.2	4.3	0.70	0.851
Combined midside	2.9	3.6	0.27	0.011

The estimates of blood flow through the midside region from Experiments 3.1 and 3.2 were combined and the ANOVA indicated that there was no difference in the rate of blood flow from each trial (P = 0.948) nor was there any interaction between strain and trial (P = 0.181). This enabled the results for the two groups of sheep to be amalgamated, resulting in eight sheep representing each strain. There was a significant difference in the

rate of blood flowing through the skin of the midside between the two strains (P = 0.011; Table 3.5).

3.3.5. Wool growth, follicle density and blood flow

Wool growth within the midside patch differed significantly between the two strains of Merinos during the period prior to blood flow measurements (P < 0.001; Table 3.6). The density of follicles in the midside and in the abdominal flank did not differ between the strongwool and finewool Merinos, nor was midside follicle density correlated with wool production rates (r = 0.292; P > 0.1). The densities of the follicles in the two regions were correlated (r = 0.806; P < 0.02).

Table 3.6. Wool production rates (mg/cm^2/d) on the midside patch and follicle density (FD; per mm^2) of both the midside and abdominal flank in finewool and strongwool Merinos (means with standard error in parentheses)

	Finewool	Strongwool	*P* <
Wool growth	0.47 (0.046)	0.96 (0.093)	0.001
FD - midside	42.3 (3.69)	52.0 (3.87)	n.s.d.
FD - flank	48.7 (7.62)	53.5 (6.48)	n.s.d.

Wool growth on the midside was significantly correlated with blood flow through the skin when the two trials were combined (r = 0.58, P < 0.01; Fig. 3.4). The relationship between blood flow and wool growth was significant within the finewool Merinos but not the strongwool Merinos. The ß-coefficients were similar for both strains (Equations 5.2 and 5.3). Blood flow and follicle density was not correlated (r = 0.44; P < 0.1) when the data from both the midside and abdominal flank were analysed (Fig 3.5).

Equation 5.2. (Finewool Merino)

$y = \mathbf{0.110x} + 0.16$ (r = 0.76; P < 0.05)

Equation 5.3. (Strongwool Merino)

$y = \mathbf{0.120x} + 0.48$ (r = 0.23; P > 0.1)

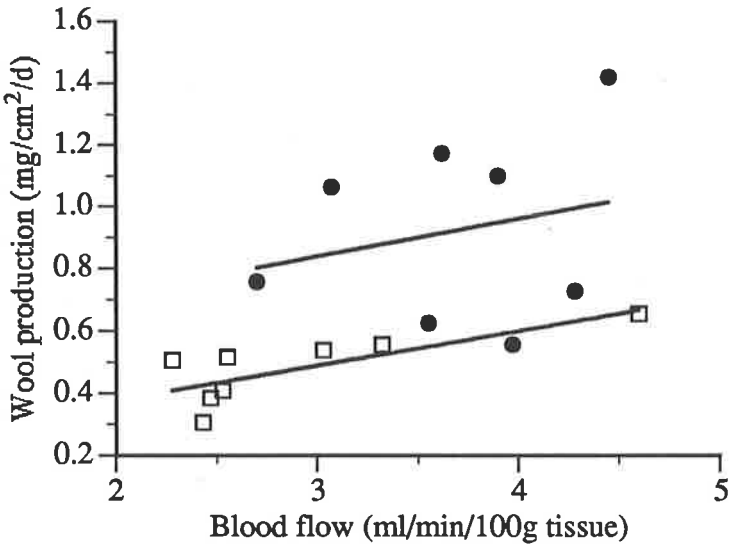

Figure 3.4. Relationship between blood flow through the skin and wool production on the midside in finewool (□) and strongwool Merinos(●) [r = 0.58; P < 0.02].

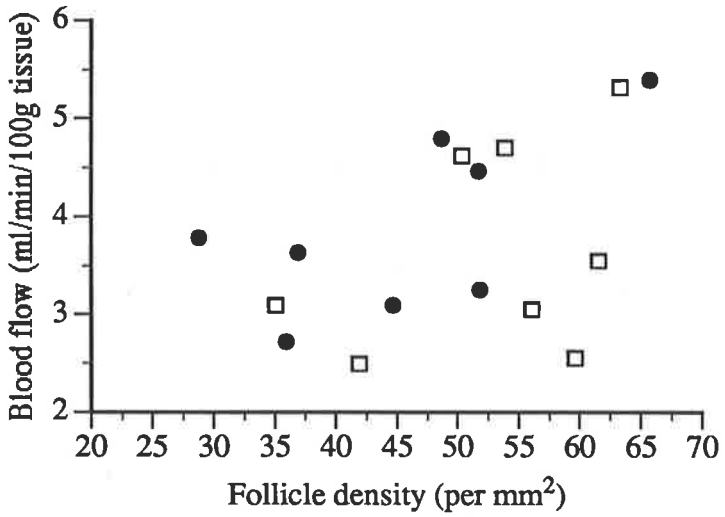

Figure 3.5. Relationship between follicle density and blood flow through the skin of the midside and abdominal flank in finewool (□) and strongwool Merinos(●) [r = 0.44; P < 0.1].

3.4. DISCUSSION

The present study was the first to examine the relationship between wool growth and blood flow through the skin of two strains of Merino. The results support the hypothesis that differences in wool growth between strongwool and finewool Merinos, and even within strains, are associated with differences in blood flow through the skin. This is in agreement with recent results from Hales and Fawcett (1993) who found a difference in blood flow per unit area of skin between the Fleece-plus and Fleece-Minus flocks. The present results indicated that a 100% increase in blood flow through the skin was associated with a 150% increase in wool growth. This is a far greater response than that calculated by Black and Reis (1979) in their model. They predicted that a 100% increase in the rate of blood flowing through the skin would result in a 70% increase in wool production due to an increase in the supply of sulphur amino acids (assuming that the release from digestion and metabolism is not rate limiting) and a resultant decrease in follicle bulb turnover time. This model was a simulation for increased blood flow in a 'standard' sheep, i.e. blood flow was increased and other factors remain the same (i.e. factors such as follicle density, number of cells in the bulb, the proportion of bulb cells which enter the fibre and the final mass of the cortical cells), rather than a comparison between sheep. This may explain the discrepancy. To conclude, then, from the present results that increases in blood flow **cause** a greater rate of wool production between strains of Merinos is premature since only 34% of the variance in wool growth was accounted for by blood flow but it appears that blood flow through the skin may be related to wool production in Merinos.

There was no overall statistical difference in blood flow through the skin at different sites within the midside region but there was often large variation between sites-within sheep, in agreement with results of Tenland *et al.* (1983) and Braverman *et al.* (1990). The latter found a 100% variation in red blood cell flux as the LDV probe was moved over a distance of 2 to 6mm. The variation was attributed to the position of the probe in relation to underlying ascending arterioles and their branches and this was likely to have been the scenario in the present study. The mean of multiple readings over an area was used to provide an average rate of blood flow in the present study, as reported by others (Holloway & Watkins, 1977; Klemp & Staberg, 1985; Monteiro-Riviere *et al.*,

1990). In the present study, wool growth was estimated over an area of skin, thus it is valid to obtain a mean blood flow rate from the same area.

There was a difference in blood flow to the skin of the midside and abdominal flank regions. This result is in agreement with the findings of those who have compared blood flow in different regions of sheep skin (Setchell & Waites, 1965; Hales & Fawcett, 1992), those who have used skin temperature as an indicator of skin blood flow in sheep (Cockrem & Wickham, 1960) and those who have compared cutaneous blood flow between different regions in other species (Lundberg & Smedegard, 1981; Tur *et al.*, 1983; Sundberg, 1984; Monteiro-Riviere *et al.*, 1990). There was a greater rate of blood flow through the skin of the abdominal flank region than through the midside in the present study, a finding which is in contrast to the presence of a dorso-ventral and anterior-posterior gradient in wool growth rates (Henderson, 1953; Chapman & Young, 1957; Young & Chapman, 1958) and in blood flow (Hales & Fawcett, 1992) in sheep. The discrepancy may have been due to movement of the sheep which artificially increased the LDV measurements since the region measured in the abdominal flank underwent strong respiratory movements in contrast to the midside, which was comparatively stable. This movement artifact has been reported elsewhere (Tenland *et al.*, 1983) so most workers restrain or train the subjects to remain motionless during the measurement period to improve the stability of readings (Svensson & Jonsson, 1987; Thomsen *et al.*, 1988; Kastrup *et al.*, 1989; Monteiro-Riviere *et al.*, 1990). Often subjects who are too restless are withdrawn from studies (e.g. Klemp & Staburg, 1985). This movement artifact was a problem and possibly contributed to the high coefficient of variation recorded in Experiment 3.2 and resulted in only midside data being used for the present analyses. It was decided not to use pharmacological agents to restrain the movement of the animals in the present work since it has been reported that anaesthesia decreases cutaneous blood flow in sheep (Weaver *et al.*, 1990).

The absolute values of cutaneous circulation obtained using LDV in unanaesthetised sheep in a thermoneutral environment were significantly lower than those obtained using radiolabelled microspheres in both the present study and in studies reported elsewhere (Table 3.7; Alexander *et al.*, 1973; Hales, 1973a, 1974; Hales *et al.*, 1976; Bell *et al.*, 1983). However, there are important differences between the two techniques. In particular, the LDV measures the velocity and number of red blood cells moving through

the circulatory system whereas the microsphere technique measures the rate at which the microspheres become lodged in blood vessels with a diameter of 15μm in the skin. If capillaries branch at diameters less than 15μm, there may be a change in the rate of blood flowing through these smaller vessels but this cannot be measured by the technique using 15μm microspheres. It is these smaller vessels which are likely to be situated in and around the follicular region, particularly through the dermal papilla. There are no reports pertaining to capillary size in the skin of sheep, but it is known that microcirculatory vessels in skin can be smaller than 15μm, for example human skin has vessels with an outside diameter of 7.5μm to 35μm (reviewed by Braverman, 1989) and the hairless mouse ear has vessels ranging from 4μm to 13μm in diameter (Eriksson *et al.*, 1980; Barker *et al.*, 1989; Mayrovitz, 1992).

The present LDV data suggests that the blood flow through the vessels surrounding the follicles was less than that estimated to flow to the skin by the microsphere methods. Nevertheless, the two methods were significantly correlated, thus measurements made using LDV give a relative rate of blood flow and are valid, as are comparisons between animals using the same method. A number of studies have been undertaken to compare the rate of blood flowing to/through the skin determined by LDV with that determined by alternative methods. Generally, there is a good relationship between blood flow rates estimated with LDV and with plethysmography (Tur *et al.*, 1983; Johnson *et al.*,1984; Wester *et al.*, 1984; Saumet *et al.*, 1986), video microscopy (Tooke *et al.*, 1983; Tyml & Ellis, 1985), heat thermal clearance method (Saumet *et al.*, 1986), blood flow meters (Pershing *et al.*, 1989) and ^{133}Xenon clearance (Holloway & Watkins, 1977; Engelhart & Kristensen, 1983). However relationships between LDV and some of these techniques are not always satisfactory (Klemp & Staberg, 1985; Kastrup *et al.*, 1987; Svensson & Jonssön, 1987; Engelhart *et al.*, 1988) and it was suggested that this was due to the techniques measuring different parameters. For example, various methods measure cutaneous blood flow at different depths (Saumet *et al.*, 1986) and other methods measure different types of blood flow*. It was suggested that LDV measures total cutaneous circulation, whereas the

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* Skin blood flow is composed of two components - a nutritional and a non-nutritional component, with changes in the non-nutritional flow acting as a thermoregulatory mechanism. Much of the non-

^{133}Xenon washout technique only measures the nutritional component of the circulation. The conclusion that LDV measures both nutritional and non-nutritional circulation was supported by the present data (Experiment 3.1), since temperature and relative humidity were correlated with blood flow estimates, indicating measurement of thermoregulatory circulation. Ostergren and Fagrell (1986) reported that the velocity of red blood cells in cutaneous capillaries is increased with increased skin temperature in humans which may indicate a change in nutritional flow with temperature. This is contrary to the theory that thermoregulatory mechanisms do not operate at the capillary level, thus LDV may measure some temperature effects not related to thermoregulation.

Table 3.7. Rate of blood flow to the skin of sheep in a 'thermoneutral' environment estimated using various techniques

Method	Position	Blood flow (ml/min/100g tissue)	Reference
LDV	Midside	4	Present (Exp 3.3)
15µm microsphere	Midside	10	Present (Exp 3.3)
^{86}RbCl & 4-^{131}I-iodoantipyrine	Midside	12	Setchell & Waites, 1965
^{86}RbCl	Lamb / Midside	25	Alexander *et al.*, 1973
15µm microsphere	Midside	18	Hales, 1973a
15µm microsphere	Midside	12	Hales, 1974
15µm microsphere	Hind limb	2 - 7	Hales *et al.*, 1978b
A-V difference*	Abdominal flank	6 - 9	Harris *et al.*, 1989
15µm microsphere	Midside	11	Hales & Fawcett, 1992

* Arterio-venous difference

circulation is shunted through cutaneous arteriovenous anastomoses (AVAs) which provide direct communication between arterioles and venules, serving no function in tissue metabolism. Conversely, the nutritional component provides the transport of nutrients to tissues for metabolism and removes metabolic waste products. The nutritional component of skin blood flow is small when compared with the maximum total flow available in the cutaneous circulation.

3.4.1. Conclusion

Laser Doppler velocimetry has advantages over other methods in that it provides a non-invasive and continuous means of measurement of blood flow through the skin. In particular, in the measurement of cutaneous circulation in sheep, it is preferable to other methods because there are no problems associated with the handling and disposal of isotopes, there are no surgical and post-surgical complications as experienced with the microsphere technique in the present study and ultimately it is not necessary to slaughter the animals to obtain samples for analysis. The LDV technique enables cutaneous circulation to be readily measured in sheep in a range of circumstances and will facilitate further research into the physiology of wool growth. For example, it will be possible to examine the effects of blood flow on wool production in relation to staple strength, seasonality and physiological state.

Using the LDV technique, it was found that blood flow through the midside skin of sheep differs between strains of Merinos and is associated with rate of wool production as was initially hypothesised. However, the possible cause of the difference in blood flow between strains is unknown and is examined in Chapter 4.

Chapter 4.

Cutaneous microvasculature of finewool and strongwool Merinos

CHAPTER 4. CUTANEOUS MICROVASCULATURE OF FINEWOOL AND STRONGWOOL MERINOS

4.1. INTRODUCTION.

In the preceding experiments (Chapter 3) it was demonstrated that there was a difference between strains of Merino in the rate of blood flowing through the skin and that blood flow was correlated with follicle density and wool growth per unit area of skin. However, the cause of this difference in blood flow rate is unknown. The laser Doppler velocimeter used to estimate cutaneous circulation measures the velocity and total number of red blood cells moving through the vessels directly under the probe. It follows that variation between sheep in blood flow rate may be due to a difference in the velocity and/or the number of red blood cells which, in turn, may be determined by the number and/or size of the vessels under the probe in the skin. For example, the velocity of the red blood cells of the two strains of Merinos may be equivalent, but if there are more or larger vessels in the skin of the strongwool Merinos more cells may pass through the laser beam, thus giving a higher blood flow rate. The possible difference in the number and size of blood vessels in the skin of sheep is examined in this chapter.

The microvasculature of the skin of sheep has been described in detail by Ryder (1955a, b) in which three vascular levels, the subepidermal, mid-dermal and dermal nets, are recognised in a number of English long- and short-wool breeds of sheep. The networks have also been identified in the Merino (Nay, 1966). There are differences between breeds of sheep in the distribution of the vessels within the skin, for example the network is poorly developed in the Suffolk, Romney, Border Leicester, Masham and Swaledale, in contrast to the presence of extra layers of horizontal vessels in the Welsh Mountain breeds (Ryder, 1955a). Even within a breed such as the Merino, there are differences in the arrangement of the microvasculature in the skin. Nay (1966) reported the presence of a regular vascular arrangement associated with straight follicles in high-wool producing Merinos in contrast to an irregular vascular arrangement associated with tangled follicles in Merinos selected for low levels of wool production.

The literature supports the proposal that differences in the rate of blood flowing through the skin may be due to differences in the density of vessels in the skin. Furthermore, Durward and Rudall (1949) found that the density of blood vessels associated with hair follicles in the rat varies according to the stage of the hair growth cycle, suggesting a relationship between hair growth and blood vessel distribution. The present study was undertaken to test two hypotheses: firstly, that differences in the rate of blood flowing through the skin of two strains of Merinos are due to differences in the quantity of vascular tissue in the skin; and, secondly, that the quantity of vascular tissue in the skin is correlated with wool growth rate.

4.2. EXPERIMENTAL PROCEDURE

4.2.1. Experimental design (Experiment 4)

The sheep, housing conditions and blood flow measurements were the same as those used in Experiment 3.2 (Section 3.2.1.2.). Briefly, four finewool and four strongwool Merinos were housed indoors in individual pens for 46 days during which wool growth estimates were made in the midside region (Section 2.2.3.). The sheep were then transferred to a climate-control room (temperature - 18°C; relative humidity - 71%; light between 0830 and 1930) and blood flow through the skin of the abdominal flank was estimated using the laser Doppler velocimeter (Section 3.2.2.1.) on two consecutive days.

The isolated cutaneous patch preparation (Zoltie *et al.*, 1988; Harris *et al.*, 1989) was utilised to infuse blood vessels in the skin of the abdominal flank with a silicone rubber compound (Microfil®; Flowtek Inc., Boulder, USA), as described in Section 4.2.2. This was performed the day immediately after the final blood flow measurements were made in six of the sheep, and the following day in the final two sheep. Follicle density was determined from skin samples taken from the excised skin containing the infused Microfil®. This skin was processed, embedded, sectioned at 8μm transverse to the follicle and stained with haematoxylin and eosin (Section 2.2.4.1.). The number of follicles per unit area was estimated with a light microscope at a magnification of 379 times using these skin sections and Equation 2.2.

4.2.2. Isolated cutaneous patch surgery

Surgery was performed (Section 3.2.2.2.) in a non-sterile environment to isolate the deep circumflex iliac artery and vein as described in detail by Zoltie *et al.*, (1988) and Harris *et al.*, (1989). Briefly, the sheep were placed in a lateral recumbent position and a dorsoventral incision was made below the tuber coxae along the cranial border of the tensor fasciae latae muscle. The contour of the muscle was followed closely using blunt dissection to locate the deep circumflex iliac vessels and the muscle was retracted to expose the prefemoralis lymph node and blood vessels of interest. Commercial 18G catheters with 20G needle (Surflo; Teremo, Tokyo, Japan) were inserted firstly into the deep circumflex iliac artery and then into the vein and each was tied into place with silk thread.

4.2.3. Microfil® technique

The artery was perfused with heparinised saline (10 i.u./ml; saline and heparin sodium [5000U/ml] both are products of Commonwealth Serum Laboratories, Melbourne, Vic.) to flush the blood from the isolated patch. The Microfil® solution was prepared (53% diluent, 42% Microfil, 5% curing agent; all supplied by Flowtek Inc., Boulder, USA) immediately preceding infusion. Hand-pressured syringes were used to administer 10ml of Microfil® firstly into the circumflex iliac vein and then into the corresponding artery with a 15 minute break between the two administrations to allow the Microfil® to set. Orange and blue Microfil® were used to enable differentiation between the arteries and veins, with orange being infused into the arteries and blue into the veins of six sheep and *vice versa* in the remaining two sheep. After the infusion, the sheep were slaughtered with an overdose of pentobarbitone (Nembutal®; Boehringer Ingelheim, Artarmon, NSW) and then stored in a cold room overnight to allow the Microfil® to cure.

The area of skin perfused with the Microfil® was sketched and then removed from the sheep. The samples were dehydrated through 25%, 50%, 70% and 95% ethanol (v/v; 24 hours each) and stored in 100% ethanol. To visualise the perfused blood vessels, the skin was cut into strips in ethanol with two scalpel blades held 1mm apart, and then cleared in methyl salicylic acid (Ajax Chemicals, Auburn, Australia). The cleared skin strips were placed on a microscope slide, mounted with D.P.X., coverslipped and examined

immediately. The area of vascular tissue in the skin (mm^2 of blood vessels per mm^3 of skin) was estimated using a video counting and microdensitometry computer program (BQ System IV; R&M Biometrics, Tennessee) at 159 times magnification. The program provides a semiautomated analysis of video images. Briefly, the skin is viewed by video through a microscope and displayed on a screen (Plate 4.1a). The screen image is subdivided into a matrix of small dots (pixels), the brightness of each pixel is determined and then stored as a digital byte in the computer's memory. The computer is then asked to select the pixels which fall in a certain range of brightness corresponding to the blood vessels in the skin (Plate 4.1b).. This can then be described as the area of blood vessel per unit volume of skin (BVI - blood vessel index). The latter is estimated from the total area of skin analysed, multiplied by 1mm (the width of skin section).

4.2.4. Statistical analysis

The mean ± standard deviation were routinely calculated from sheep-within strain data. The BVI was estimated on two separate days and the data from the two days were combined. Students t-tests were used to compare wool growth, follicle density, blood flow and BVI between strains.

Correlation coefficients were estimated with the Cricket Graph computer software package (version 1.3.1; Cricket Software, Philadelphia, USA). This was used to determine the relationships between BVI and wool growth, follicle density and blood flow.

Results were considered significantly different when $P < 0.05$.

4.3. RESULTS

4.3.1. Blood vessel distribution

The Microfil® perfusion through the abdominal flank was successful in all sheep. The Microfil® perfused through the artery was visible whereas the colour perfused through the veins was never observed either superficially (Plate 4.2a,b) or after the skin was removed from the sheep. The perfused area varied considerably between sheep (Fig. 4.1.) but the distribution of Microfil® did not appear to be related to strain, BVI or wool growth.

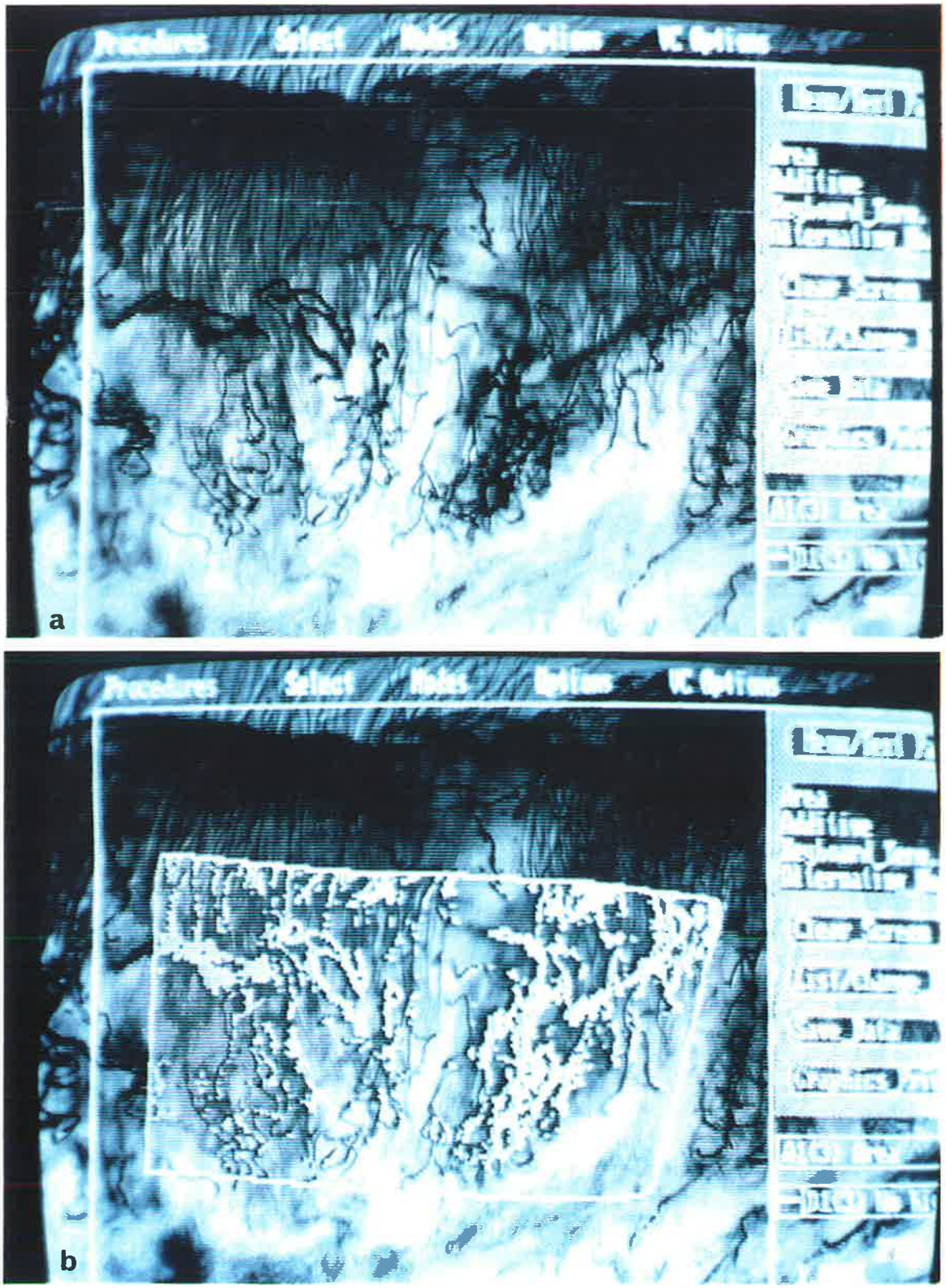


Plate 4.1. Photomicrograph of blood vessels in the skin filled with Microfil, projected onto a video screen.

a) Video image of the blood vessels.

b) Image analysis of the vessels in a defined area. The area of vascular tissue per unit volume of skin was determined by a software program which estimated the amount of tissue which fell inot a defined level of brightness (that which is highlighted in the above image).

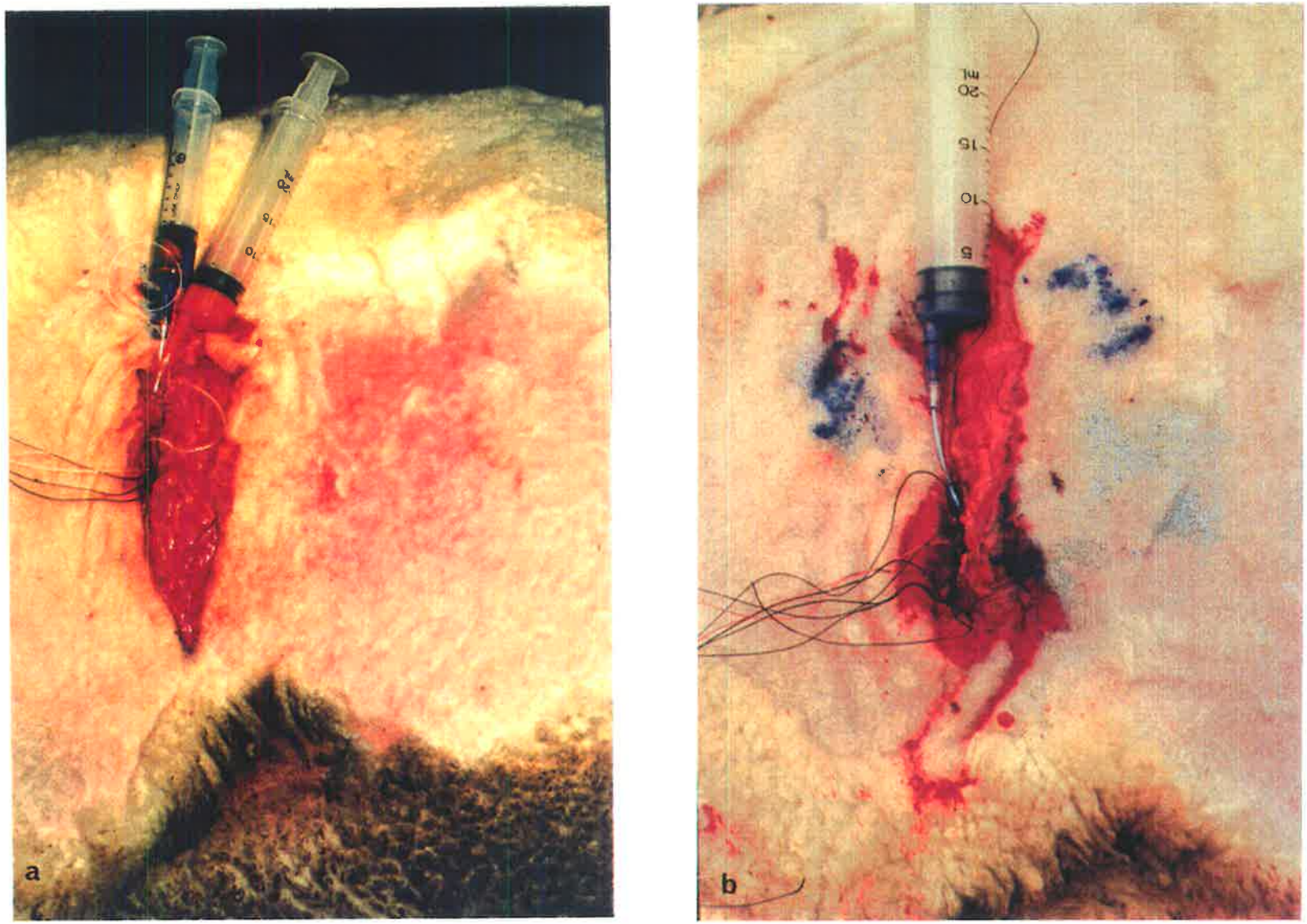


Plate 4.2. Skin perfused with Microfil® which is visible cranial to the incision.

a) Orange Microfil was perfused into the deep circumflex iliac.

b) Blue Microfil was perfused into the deep circumflex iliac.

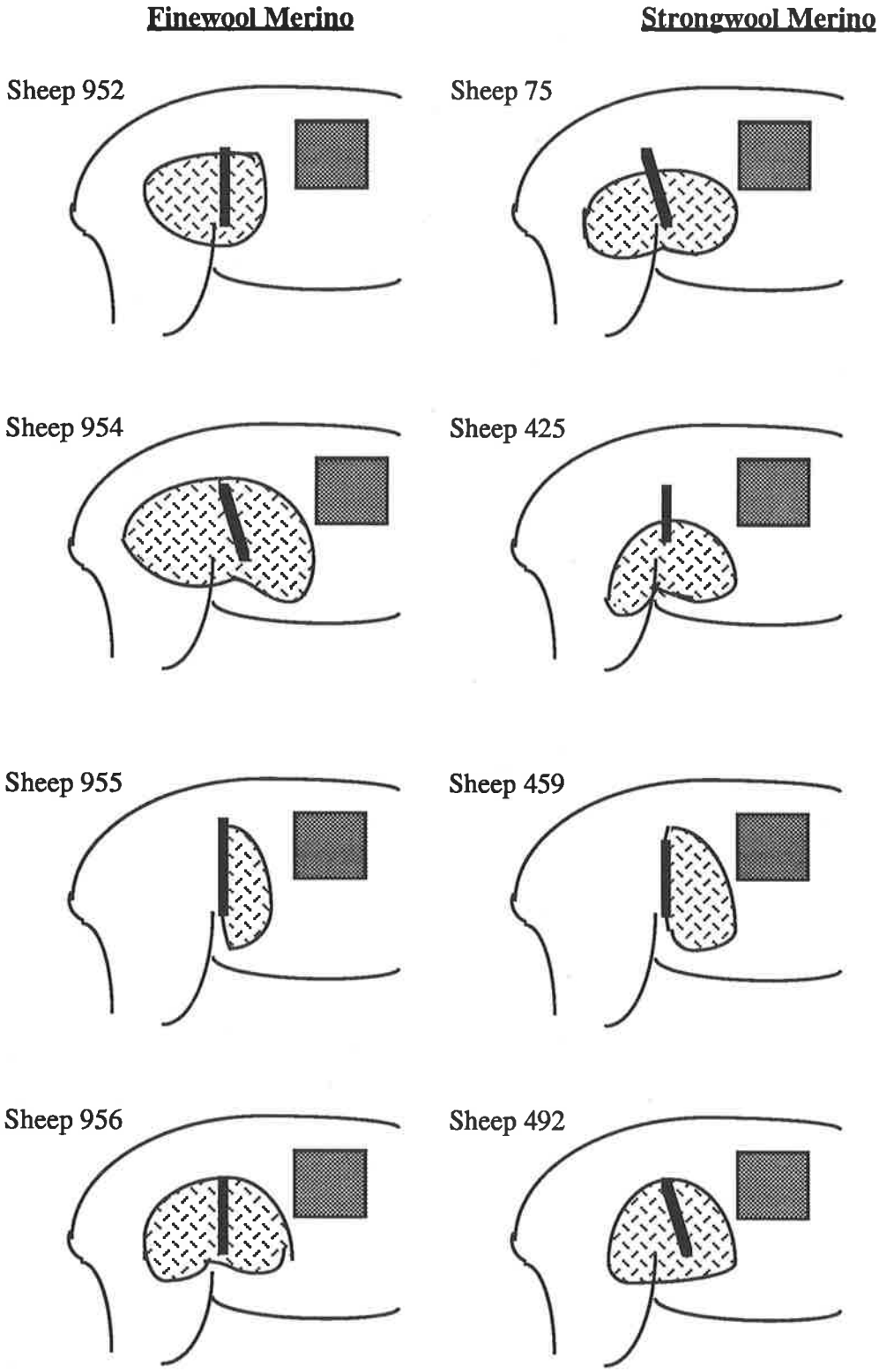


Figure 4.1. The area perfused by Microfil® in the abdominal flank region of the strongwool and finewool Merinos , in relation to the surgical incision , and the midside patch .

Within the skin samples cleared with methyl salicylate, the perfused blood vessels were the only structures clearly visible, although it was sometimes possible to discern the outline of the follicles depending on the degree of tissue clearing (Plate 4.3). There appeared to be a delineation between the networks as described by Ryder (1955a) and Nay (1966) with the dermal network being discrete from the remainder of the vessels (Plate 4.4a). It was impossible to separate the mid-dermal and sub-epidermal networks, but it was obvious that the vessels surrounding the follicles originated from these regions. Blood vessels were occasionally observed in the dermal papilla in the strongwool Merinos (Plate 4.3).

4.3.2. Area of vascular tissue per unit area of skin

The index of the amount of vascular tissue in the skin (mm^2 of blood vessel per mm^3 of skin; BVI) varied considerably between sheep (Table 4.1.) but did not differ between the two strains of Merinos. There was also a large variation within each sheep (Table 4.1.) with the CV ranging from 48% to 100%. This was due to many areas within the skin not having any perfused vessels and other areas where the density of the vessels were very high. Plate 4.4b shows this occurrence within a single region of a skin sample.

Variation in skin blood flow between sheep could not be explained by the relative amount of vascular tissue present in the skin, in fact it accounted for only 0.2% of the variance in skin blood flow, as indicated in the graph of blood flow and vessel density (Fig 4.2). Likewise there was no relationship between follicle density and vessel density.

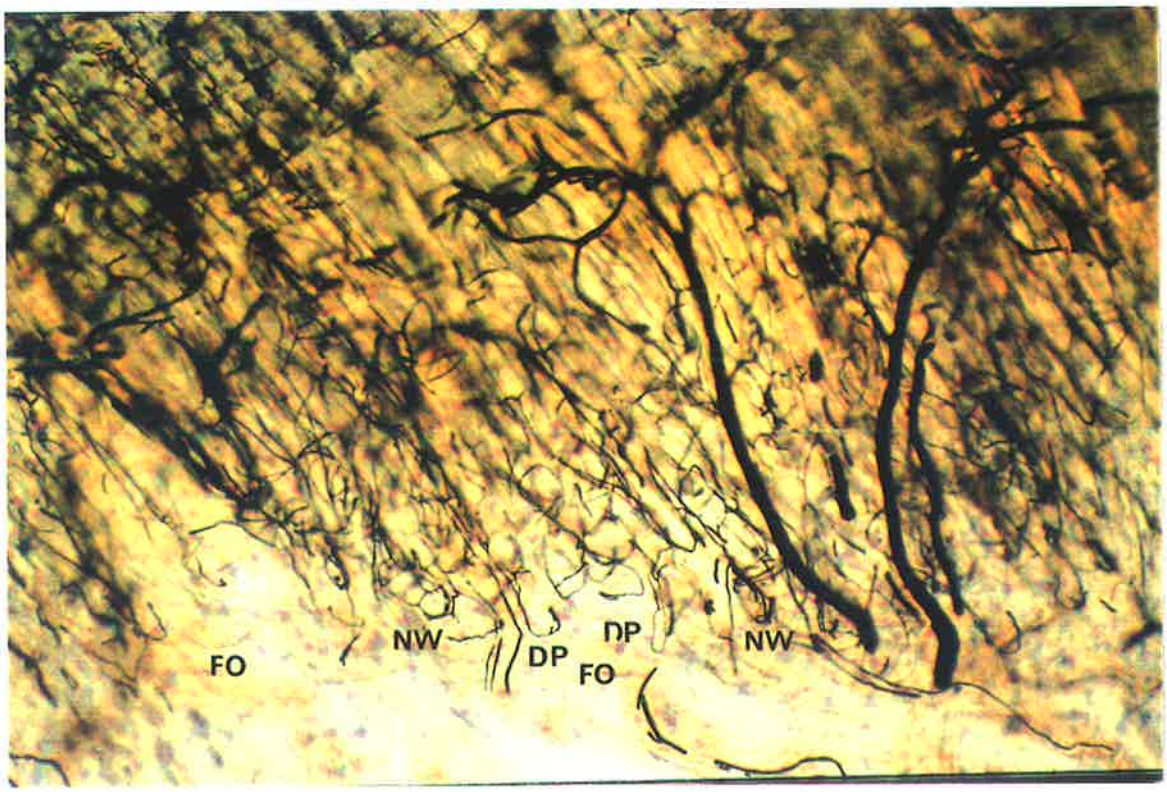


Plate 4.3. Photomicrograph of blood vessels perfused with Microfil® in the skin of sheep. 1mm thick sections, magnification 43x.
FO - Follicle outline
DP - Blood vessel in dermal papilla
NW - Network of vessels around follicle.

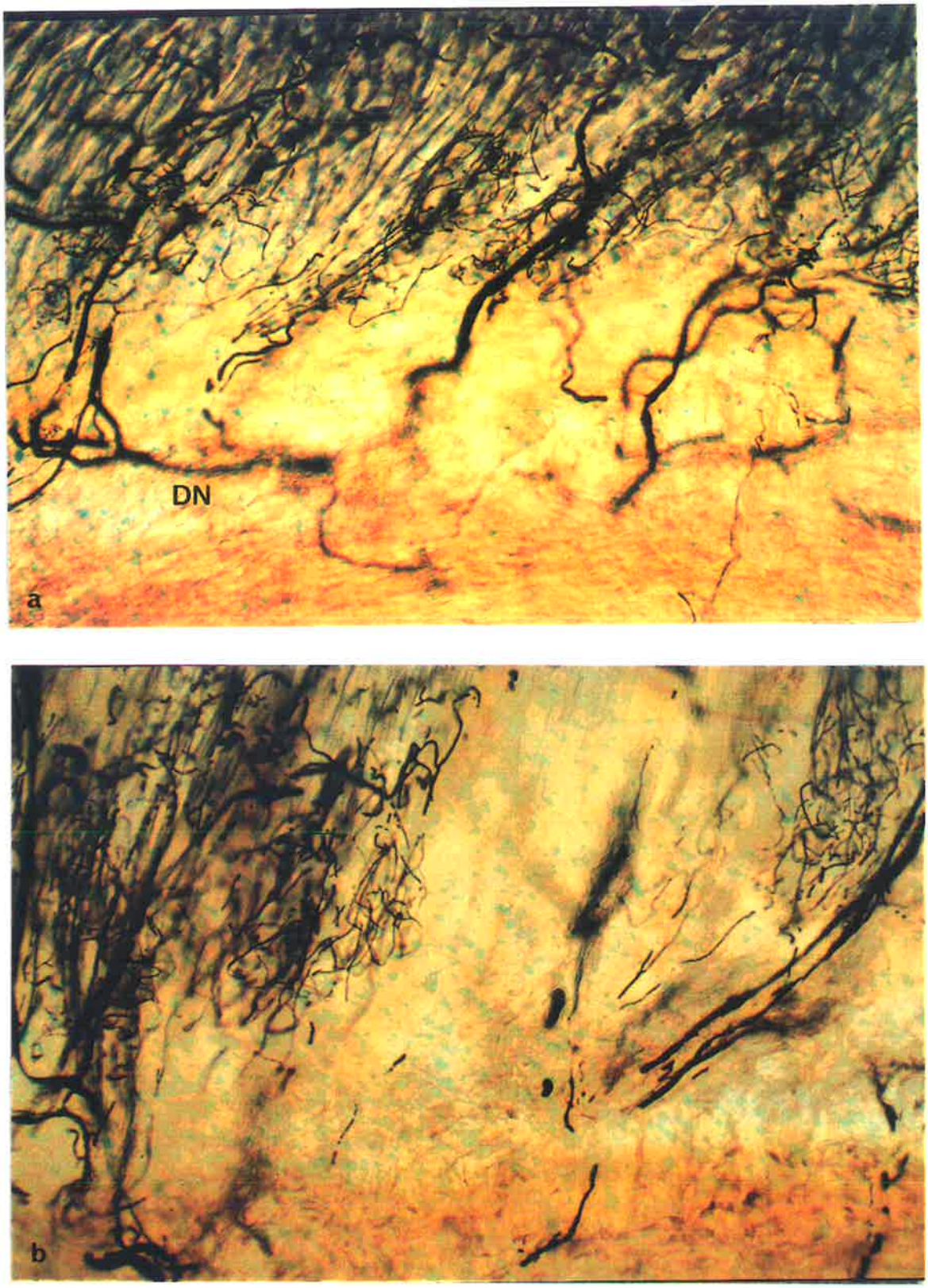


Plate 4.4. Photomicrographs of blood vessels perfused with Microfil® in the skin of sheep. 1mm thick sections; Magnification 43x

a) Dermal network in the skin **(DN)**

b) Perfused and non-perfused regions within the skin.

4.3.3. Wool growth correlates

There were no significant correlations between wool growth on the midside and blood flow, BVI or follicle density, nor were there any significant relationships between any of the other characteristics with the exception of a correlation ($P < 0.02$) between follicle density in the midside and in the abdominal flank (Table 4.2).

Table 4.1. Midside wool growth rates (wool; mg/cm^2/d), and follicle densities (FD; per mm^2), blood flow rates (flow; ml/min/100g tissue), and index of the area of vascular tissue in the skin (BVI; mm^2 of blood vessel/mm^3 of skin) from the abdominal flank region, coefficient of variation of the index (CV; %) and the number of estimates of the area of vascular tissue (n=) in finewool and strongwool Merinos (means with standard deviation in parentheses)

Sheep No.	Wool	FD	Flow	BVI	CV	n =
Finewool						
952	0.65	53.9 (4.33)	4.7 (1.91)	0.07 (0.07)	100	266
954	0.38	35.1 (2.46)	3.1 (0.71)	0.10 (0.07)	70	304
955	0.53	61.6 (3.57)	3.5 (1.01)	0.17 (0.13)	76	293
956	0.40	63.3 (4.15)	5.3 (3.23)	0.25 (0.12)	48	349
mean	0.49 (0.054)	53.5 (6.46)	4.2 (0.51)	0.15 (0.035)		
Strongwool						
BT	1.06	48.7 (3.99)	4.8 (1.93)	0.07 (0.06)	86	302
75	1.41	65.7 (4.73)	5.4 (1.74)	0.15 (0.11)	73	305
425	0.75	28.8 (1.58)	3.8 (2.35)	0.20 (0.11)	55	304
459	1.17	51.8 (3.79)	3.2 (0.93)	0.18 (0.12)	66	295
mean	1.10 (0.119)	48.8 (7.61)	4.3 (0.42)	0.15 (0.029)		
P<	0.01	> 0.05	> 0.05	> 0.05		

Table 4.2. Table of relationships between various characteristics including midside wool growth rates (wool; mg/cm^2/d) and follicle density (msFD; per mm^2), and the follicle densities (afFD; per mm^2), blood flow rates (flow; ml/min/100g tissue) and index of the amount of vascular tissue in the skin (BVI; mm^2 of blood vessel/mm^3 of skin) from the abdominal flank region (Correlation coefficients)

r =	Wool	msFD	Flow	BVI
Wool	1.000	-0.292	0.141	0.395
afFD		0.806	0.586	0.184
Flow			1.000	0.045
BVI				1.000

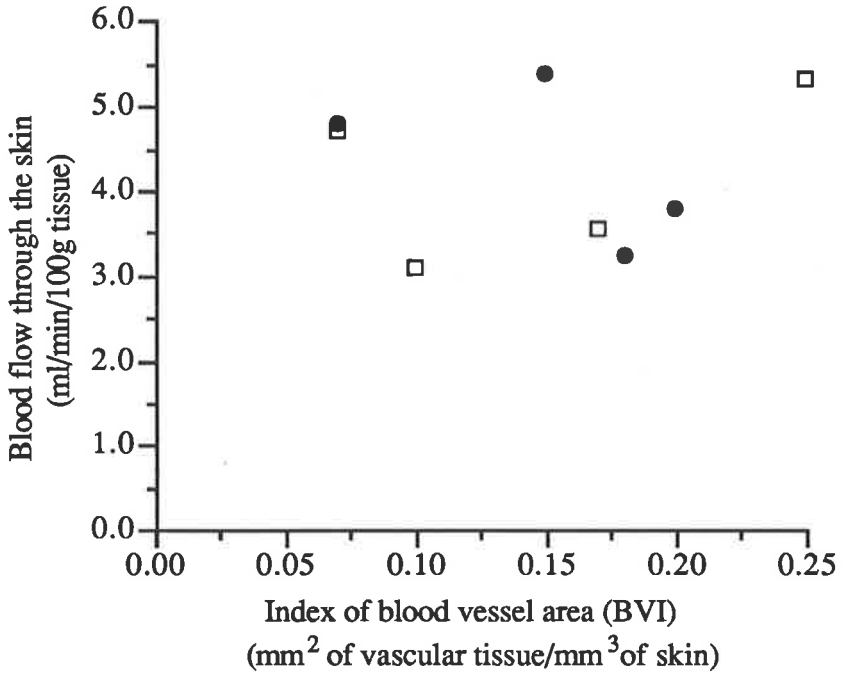


Figure 4.2. The relationship between the area of vascular tissue per unit volume of skin and the amount of blood flowing through the skin in the abdominal flank region of finewool (□) and strongwool (●) Merinos

4.4. DISCUSSION

The experiment reported in this chapter is the first to analyse the area of vascular tissue per unit volume of skin in sheep. Others have reported the arrangement of blood vessels in different breeds of sheep (Ryder, 1955a) and strains of Merinos (Nay, 1966) but not the 'amount', *per se*. The present experiment used an index of the quantity of blood vessels (BVI) which described the area of blood vessels per unit volume of skin (i.e. mm^2 of blood vessels/mm^3 of skin). This index was the same in the finewool and strongwool Merino and had a similar range within the two strains (Table 4.1.). The large variation in BVI within the two strains indicated that it was unlikely there would be a difference between the two groups even if there were more sheep in each group ($n > 4$).

There was more variation in the BVI within individual sheep than between sheep since there was a very high coefficient of variation (CV) in all sheep (> 48%; Table 4.1.). Often there were areas of skin which had very few perfused vessels and no network of vessels around individual follicles. These factors contributed to the high CV. The lack of blood vessels in some areas was not unusual since Ryder (1955a,b) and Nay (1966) reported that networks of blood vessels were never seen around every follicle in the skin of adult sheep. The photomicrographs displayed in this chapter unequivocally show the absence of vessels around many follicles. A computer reconstruction of vessels present in human skin indicated areas lacking in arterioles and post-capillary vessels, although the authors did not refer to the presence or absence of hair in the regions of skin examined (Braverman *et al.*, 1990).

The BVI was not related to the rate of blood flowing through the skin, so the blood flow measurements obtained using the LDV were unlikely to have been influenced by the number of vessels underlying the probe. This suggests that differences in the rate of blood flow in the skin were not due to differences in the microvasculature of the skin, rather there may have been a variation in the number of blood cells or the velocity at which the cells were travelling. It has been reported that there is considerable variability of cell velocity in the human cutaneous capillaries both within and between individuals (Bollinger *et al.*, 1974; Fagrell *et al.*, 1977; Richardson, 1982; Ostergren & Fagrell, 1986), suggesting that the velocity of the red blood cells in the sheep may be related to differences in blood flow.

The use of Microfil® with a video microdensitometry program did not differentiate between the size and number of individual blood vessels. For example, the strongwool Merinos may have fewer, larger vessels in contrast to the finewools which may have more, smaller vessels or *vice versa*, both of which would result in the same total area of vascular tissue per unit volume of skin. If this was the case, then more red blood cells may be capable of passing through the larger vessels, thus contributing to a greater blood flow rate. The use of silicone rubber is a robust technique suitable for comparative anatomical understanding of the microvasculature of organs. The technique generally is used to define the spatial organisation of large supply and drainage vessels in both pathological and non-pathological states (Reynolds *et al.*, 1967; Reynolds, 1972; Reynolds & Swan, 1972; Swan *et al.*, 1978; Granger & Bulkley, 1981; Ohtani & Gannon, 1982; Browning & Gannon, 1984). In order to separate differences in vessel size and number, it is necessary to use a more precise technique involving the examination of vascular corrosion cast of the vessels with an electron microscope, reviewed recently by Lametschwander *et al.* (1990). The casts are made by perfusing the tissue of interest with a polymerising casting-media which can stand electron bombardment. After infusion, the area is excised and the tissue macerated to produce a cast which permits visualisation and analysis of the blood vessels. A problem associated with using this technique is in being able to select a site which enables the area of interest to be completely (and inexpensively) perfused. This is relatively easy in organs which can be isolated or have a discrete blood supply such as the uterus (Rogers & Gannon, 1983), fallopian tubes (Verco & Gannon, 1984), or in the study of whole small animals such as the rat (e.g. Ohtani & Gannon, 1982), although even the rat is often too large to perform a whole-body perfusion. Tumor tissue also has been induced to grow as an isolated organ with a single artery and vein, which permits microvascular casting at controlled vascular pressures (Less *et al.*, 1991). The injection of the compound used for corrosion casting into large animals for the study of mammalian skin is difficult and there are very few reports utilising this technique. Those who have used it, have injected the compound into an isolated region rather than the whole body, for example, in studies examining the microvasculature of the skin, the femoral artery was perfused in rats (Imayama, 1981) and the axillary artery was cannulated in the hand of the Japanese monkey (Umeda & Ikeda, 1988).

Most reports of casting techniques usually involve flushing the circulatory system with saline to remove the red blood cells prior to perfusion. In the present experiment, it was not possible to flush the 'isolated' patch due to collateral circulation. This is not unusual since it has been reported that there is a 10 - 20% loss of blood through venous drainage, particularly under extreme conditions of high blood pressure (Dellow *et al.*, 1988). This may explain the inability to flush the system in the present experiment. As the saline was being forced through the isolated patch, collateral veins opened to allow excess fluid to be removed from the area and simultaneously collateral arteries may have opened to allow the inflow of blood, although probably to a lesser extent (Dellow *et al.*, 1988).

A second problem with the technique was the variable area perfused (Fig. 4.1). This was not a problem in the present experiment but is a problem inherent in the isolated cutaneous patch technique. Similarly, the failure to detect the Microfil® perfused through the vein suggests that the iliac vein was not solely draining the cutaneous area. Attempts were made to identify the venous-perfused region but these were unsuccessful. To conclude, the problems inherent in the isolated cutaneous patch technique were numerous but were able to be overcome in the present experiment. However, care must be taken in future when interpreting results obtained using this technique.

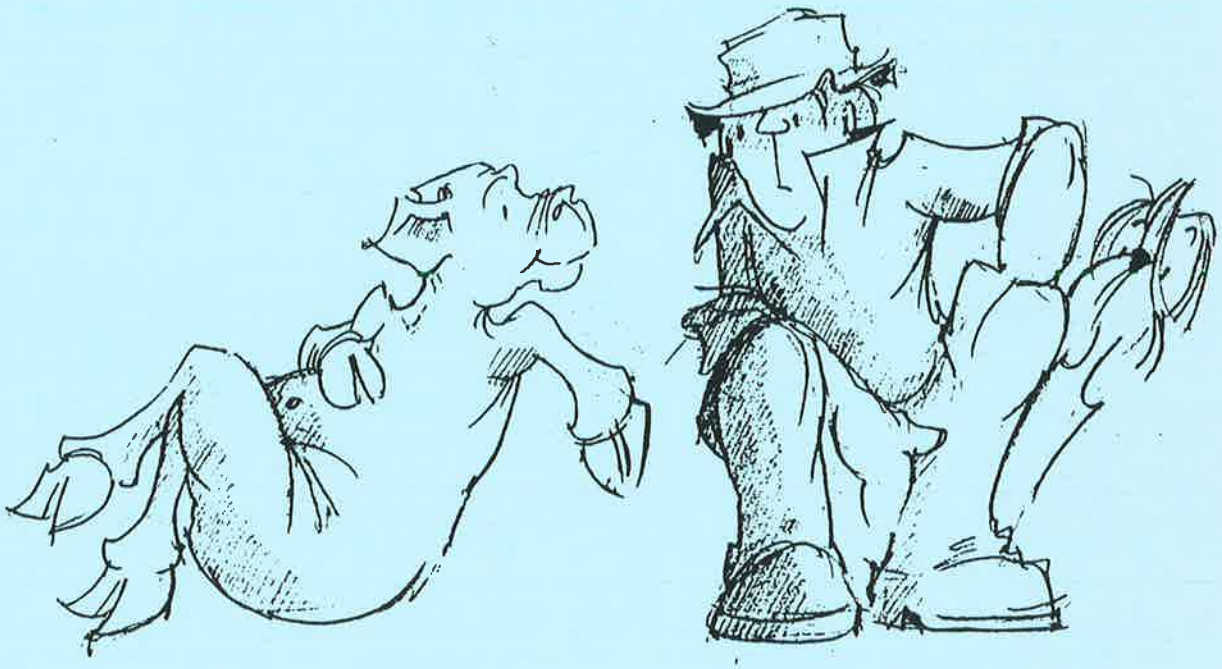
The BVI was not associated with wool growth or follicle density and it appears that BVI is independent of skin and follicle functioning. Nay (1966) found that the blood supply pattern could be used to identify individual primary follicle groups, suggesting that the distribution of the blood vessels is related to primary follicle density or follicle groups. He reports that individual follicle groups aggregate to form distinct anatomical units referred to as 'fields'. He reports that the blood vessels are associated with these 'fields' of follicles and the major supply and drainage vessels are located at the junctions between the fields (Nay, 1966). Below the sebaceous glands, the vessels divide into horizontal or oblique branches providing supply and drainage for individual follicle groups in agreement with Ryder (1955a) and the present work.

Similar patterns of arrangement were seen in the present work between the strongwool and finewool Merinos as reported by Nay (1966), however these arrangements were not related to wool growth or fibre diameter, even within strains. It is concluded from

the present work that there was no relationship between the area of cutaneous blood vessel and wool growth, follicle density nor the rate of blood flowing through the skin and the hypothesis that differences in the rate of blood flowing through the skin of two strains of Merinos are due to differences in the area of vascular tissue per unit volume of skin was not supported. BVI may be related to the thermoregulatory requirements of the animal, or may be entirely random. Thus it is clear that much work remains in developing techniques to determine the role, if any, of the amount of vascular tissue in the skin of sheep.

CHAPTER 5.

IN VITRO UPTAKE OF NUTRIENTS BY THE SKIN AND FOLLICLES OF FINEWOOL AND STRONGWOOL MERINOS



CHAPTER 5. *IN VITRO* UPTAKE OF RADIOLABELLED NUTRIENTS BY THE SKIN AND FOLLICLES OF FINEWOOL AND STRONGWOOL MERINOS

5.1. INTRODUCTION

There is unequivocal evidence that sheep with high levels of wool production per unit body weight or per unit area of skin have a higher efficiency of conversion of feed to wool than low-wool producers (Section 1.2.2.). This difference in efficiency is not due to variability in feed intake, nor digestive efficiency (Section 1.2.3). It appears that differences between sheep in wool growth are achieved despite similar quantities of nutrients entering the circulatory system of the sheep. Williams (1987) and Black (1987) suggested that efficiency of wool production may be determined by a mechanism involving the partitioning of nutrients between the skin and the rest of the body differs between sheep or that the follicles of some sheep are able to utilise the available nutrients more effectively. It was shown in Chapter 3 that there is a greater rate of blood flowing through the skin of strongwool Merinos than finewool Merinos and this was correlated with wool production levels. This implies that there would be a greater supply of nutrients to the follicles of high wool-producing sheep associated with the greater blood flow rate. Whether there is a difference between sheep in the ability of skin and follicles to absorb and incorporate nutrients from an extracellular pool has not been examined.

Previous studies indicate that the major nutritional limitation to wool growth is the amount and composition of amino acids available to the wool follicle (Reis, 1969; Kempton, 1979; Reis *et al.*, 1988; 1992). The two studies by Reis and colleagues (1988; 1992) also showed that an interaction exists between protein and energy supply. This result is in agreement with Black *et al.* (1973), who found that wool growth was slightly enhanced by energy supply at high levels of protein, but not when protein was limiting. Wool production in strongwool Merinos is associated with high rates of cell division and the maintenance of a greater volume of germinative tissue (Chapter 2), and since cell proliferation, protein synthesis and keratinisation require a larger amount of energy, follicles must have a high energy demand for follicle-cell metabolism. *In vitro* studies by

Bullough and Laurence (1958) have shown that carbohydrates are necessary for cell division to occur in mice hair. Carbohydrates may therefore play an important role in wool growth by supplying energy for metabolic processes via oxidation of glucose for the cells. The main pathway for glucose utilisation in the skin and follicles is glycolysis (Embden-Meyerhof pathway) with the majority of glucose being anaerobically oxidised to lactate and less than 10% of the utilised glucose being aerobically oxidised to CO_2 and water by the tricarboxylic acid cycle (Adachi & Uno, 1968; Philpott & Kealey, 1991). Glucose appears to be the major fuel for follicle activity since Philpott and Kealey (1991) found that fatty acids have no effect on the rate of glucose utilisation or oxidation, although they did report that follicles are capable of utilising lipids in the absence of glucose. The follicle is capable of producing energy by the oxidation of proteins and amino acids (e.g. glutamine; Kealey *et al.*, 1991). In the study by Kealey and co-workers, the follicles were incubated in greater than physiological levels of glutamine, which may have resulted in an abnormal response. The results from the current literature suggest that glucose is the major fuel for follicle metabolism and it was decided that the uptake of glucose by wool follicles should be examined in the present study.

It is generally accepted that the sulphur-amino acid cystine# plays a major role in the regulation and control of wool growth. The phenomena were initially demonstrated by Marston (1935) who subcutaneously administered cystine which stimulated wool growth and have been extensively reviewed by Reis (1979, 1989). Wool proteins are characterised by their sulphur content coming largely from cystine, with small amounts of cysteine and methionine (Reis & Schinckel, 1964; Section 1.3.3.). Thus, an understanding of the uptake of sulphur amino-acids is critical for determining the control of wool growth. Williams *et al.* (1972b) found that Fleece-plus sheep respond at a greater rate to cystine supplements than Fleece-minus sheep and it was suggested that this response may be due to differing metabolism between the genotypes. An example of possible variations in cystine metabolism is provided by the Fleece-plus sheep having a lower concentration of cystine in

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Cystine refers to both 1M cystine and/or 2M cysteine (or half-cystine) in this thesis unless the specific amino acid is intended, for example in reference to cysteine hydrochloride. Also S^{35}cysteine hydrochloride is also reffered to as S^{35}cystine.

their venous plasma than the Fleece-minus sheep (Williams *et al.*, 1972a; Williams, 1976; Williams, 1984; Williams *et al.*, 1986). Studies of cystine entry rate into the extracellular compartment have been inconclusive but tend to suggest that this factor is not related to wool growth (Williams *et al.*, 1972a; Williams, 1976; Williams & Thornberry, 1991). There was evidence in the latter work that the Fleece-plus sheep are capable of clearing a bolus dose of cystine at a greater rate than the Fleece-minus sheep. The uptake of cystine by the skin and follicles has not been examined in other Merino strains, nor is it known if there is a difference in the ability of sheep to incorporate cystine into their follicles.

This chapter describes an *in vitro* study which examines the hypothesis that differences in wool growth between sheep in similar environments are due to differences in the ability of the skin and follicles to incorporate glucose and/or cystine from an extracellular pool.

5.2. MATERIALS AND EXPERIMENTAL PROCEDURES

5.2.1. Materials

Sterile Krebs-Ringer Bicarbonate buffered solution (Krebs-Ringer) was made by mixing the following amounts of stock solutions prepared from analytical grade reagents:
160mls NaCl* (0.77M),
4.8mls $CaCl_2.2H_2O$* (0.55M),
1.6mls KH_2PO_4§ (0.77M),
160mls $NaHCO_3$# (0.154M) which was gassed for one hour,
4.8mls KCl# (0.77M), and
1.6mls $MgSO_4.7H_2O$# (0.77M). The pH was adjusted to pH 7.3 with HCl or $NaHCO_3$ at 37°C and then filter-sterilised (Sterivex-GS; Millipore, Bedford, Massachusetts). Phosphate buffered saline (PBS) was obtained from Sigma Diagnostics (St. Louis, Missouri), Williams Medium E from Gifco Laboratories (New York, New York) and Media 199 with Hanks salts and HEPES from Multicell (Cytosystems, Castle Hill, NSW).

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* Products from Ajax Chemicals (Sydney, NSW)

§ Product from Unilab By-Products and Chemicals (Alexandria, NSW)

\# Product from BDH Chemicals (Kilsyth, Vic.)

L-^{35}S-cysteine hydrochloride (114mCi/mmol), D-[6-^{3}H] glucose (23Ci/mmol) and D-[U-^{14}C] glucose (304mCi/mmol) and scintillant (ACS II) were obtained from Amersham Australia (North Ryde, NSW). Glucose (α-D+-glucose) was obtained from Ajax Chemicals (Sydney, NSW) and DL-cysteine hydrochloride, trichloroacetic acid (TClA) and glycerol (AnalR) were products from BDH Chemicals (Kilsyth, Vic.). The tissue culture wells (3424 Mark II) were products of Costar (Cambridge, Massachusetts). Lignocaine hydrochloride was purchased from Apex Laboratories (St Marys, NSW), benzalkonium (Zephiran) from Winthrop Laboratories (Ermington,NSW), saline and heparin sodium (5000U/ml) from Commonwealth Serum Laboratories (Melbourne Vic.) and Soluene 350 from Packard Instruments (Downers Grove, Illinois).

Photographic gel (L-4 Emulsion in Gel Form) was obtained from Ilford Scientific Products (Mobberley, Cheshire) and the fixer (Hypam X-Ray Rapid Fixer), hardener (Hypam X-Ray Hardener) and developer (Phenisol X-Ray Developer) were all products from Ilford (Mt. Waverley, Vic.). Haematoxylin (Harris) and DePeX were Gurr products from BDH Chemicals (Kilsyth, Vic.) and Safsolvent was a product of Ajax Chemicals (Auburn, N.S.W.).

5.2.2. Design

This chapter reports the results of three trials (trials 5a, 5b &5c) conducted prior to the actual experiment (Experiment 5) which involved strongwool (East Bungaree) and finewool Merinos. The initial three trials, each of which is described separately below, were undertaken to optimise and verify the *in vitro* techniques used for Experiment 5.

Generally, sheep were clipped, the media prepared and the pH of the media measured with a pH meter (TPS Digital pH Meter; TPS Pty, Ltd, Brisbane, Qld) the day prior to each trial. On the morning of each trial, sterile Krebs-Ringer buffer (2.3ml) and radiolabelled nutrients (0.1ml of both cystine and glucose) were aseptically dispensed into tissue culture wells and placed in an incubator (37°C; 5% CO_2 / 95% O_2). The sheep were then placed in a lateral recumbent position and the midside skin was sterilised with Zephiran and 70% (v/v) ethanol, washed with sterile saline and injected subcutaneously with a local anaesthetic (20mg/ml). Strips of skin were cut with two scalpel blades held apart by a 2mm-spacer and then the skin was removed with scissors and immediately

placed into warm media. After the skin was collected, the tissue was cut into smaller lengths (~2cm x 2mm, weighing approximately 40mg), placed into individual tissue culture wells containing the media and radiolabelled nutrients and incubated for three hours. The samples were washed in 5% (w/v) TClA for 15mins (x four times), trimmed of excess fat and wool, weighed, placed in 20ml scintillation vials, and then solubilised in Soluene 350 (0.8ml) and shaken overnight in a waterbath (60°C). After cooling, HCl (1M; 1ml) and scintillant (5ml) were added to the vials which were stored in the dark for 24 to 48 hours to decrease chemoluminescence. The samples were then placed in a ß-counter (LKB Wallac Beta counter) and the counts per minute (CPM) were recorded.

5.2.2.1. Trial 5a - Media trial

The level of nutrient uptake by skin strips from a strongwool and a finewool Merino was compared in the following four types of media (pH 7.4 - 7.5):

(i) Phosphate buffered saline

(ii) Krebs-Ringer solution

(iii) Williams Medium E

(iv) Media 199 with Hanks salts and HEPES.

Six tissue samples from each sheep were incubated in each media with ^{35}S-cystine (5µCi) and ^{14}C-glucose (5µCi) and the total quantity of radiolabelled nutrient incorporated into the skin was examined. The media in which the highest level of uptake occurred was used for the remainder of the trials. After the tissue samples were removed, each media replicate was pooled and the pH of each treatment was measured.

5.2.2.2. Trial 5b - Linearity trial

Finewool and strongwool Merino skin samples and skin used as a blank (tissue boiled in water for 10 minutes) were incubated for 30, 60, 120, 180 or 240 minutes in Krebs-Ringer solution (six replicates) with ^{35}S-cystine (5µCi) and glucose (0.67µM) or ^{14}C-glucose (0.5µCi) and cysteine hydrochloride (17.5µM). The uptake of the labelled nutrient was examined for linearity over time.

5.2.2.3. Trial 5c - Site of uptake

Skin samples from a strongwool and a finewool Merino and a blank skin sample (*vida-infra*) were incubated in Krebs-Ringer solution with ^{35}S-cystine (2μCi/ml) and glucose (0.67μM), ^{14}C-glucose (0.2μCi/ml) and cysteine hydrochloride (17.5μM) or ^{3}H-glucose (2μCi/ml) and cysteine hydrochloride (17.5μM) for 3 hours (6 replicates). Blank skin samples were incubated concurrently in the refrigerator at 4°C. After incubation and washing in TClA, three skin samples from each of the sheep and the blank were solubilised as described in Section 5.2.2 and the remaining three samples from the sheep and blank were fixed in buffered formalin (6h; Section 2.2.4.), processed, embedded and sectioned at 6μm parallel to the follicle (Appendix 1.1.). The skin sections were deparaffinised and hydrated (Appendix 1.2), and then coated with an emulsion* at 45°C, dried on ice for 10 minutes and then at room temperature for at least 120 minutes and stored in the dark at 4°C in a light-proof box with dessicant (silica gel) for 7 days (^{35}S-cystine) and up to 140 days (^{3}H- or ^{14}C-glucose). The sections were dipped in developer (diluted 1:4) for 90 seconds, rinsed in water and placed in fixer (diluted 1:4) containing hardener (diluted 1:40) for 2 minutes. The slides were then rinsed in water for 30 minutes and stained with Lille-Mayer haematoxylin# (30 sec.), differentiated with acid alcohol§ (10 sec.), dehydrated through ethanol (2 x 2min.), safsolvent (2 x 2min.) and coverslips were mounted on the slides with DePeX. The site of radiolabelled nutrient uptake was observed by microscopic examination for the presence of silver grains over the cells which had incorporated the labelled nutrient.

5.2.3. Experiment 5. Differences in nutrient uptake rates between finewool and strongwool Merinos

Four strongwool and four finewool Merinos were housed indoors in individual pens for a 190 days stabilisation period and each were fed grain-based sheep pellets (1kg/d; Milling Industries, Murray Bridge, SA). Water was available *ad libitum.* Wool growth

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* The emulsion was a mixture was 40% (v/v) photographic gel and 2%(v/v) glycerol with RO water.

\# Haematoxylin (5g), AlNH4(SO4)2.12H2O (50g), Glycerol (300ml), H2O (700ml), NaIO3 (1g), glacial acetic acid (20ml).

§ 1% (v/v); (32%) HCl in 70% (v/v) ethanol

was estimated every three to four weeks using the midside patch technique (section 2.2.3.). The *in vitro* trial was undertaken on two consecutive days with two sheep from each strain being studied per day. Enough skin was excised to provide ten replicates per sheep. The skin was incubated in Krebs-Ringer solution with L-^{35}S-cysteine hydrochloride (0.2μCi/ml) and D-[6-^{3}H]-glucose (2μCi/ml) at 37°C and the blanks for each day were incubated at 4°C. After incubation, the media for each sheep was combined and the pH of each was recorded at 37°C as described above. The radioactive content of the skin pieces was detected as described in section 5.2.2.

5.2.4. Statistical analysis

The radioactivity was expressed per unit tissue weight (CPM/mg tissue). The mean and standard error of the mean were routinely calculated with data obtained from samples within sheep and then from sheep within strains. The Students t-test was used to compare wool growth between strains in Experiment 5.

Analysis of variance statistics were performed with the Super ANOVA computer software package (1989-1990, Abacus Concepts, Inc, Berkeley, California) and was used to test the effects of media and strain on total labelled-nutrient uptake in trial 5a. The second group of analysis of variance statistics were performed with GENSTAT 5 (1988, Lawes Agricultural Trust, Rothamsted Experimental Station) which were used to examine the strain and day effect on glucose and cystine uptake by the skin and follicles in Experiment 5.

Simple correlation coefficients were estimated with the Cricket Graph computer software package (version 1.3.1; Cricket Software, Philadelphia, USA). The coefficients were used to determine the uptake of labelled-nutrients over time (trial 5b) and to derive the relationship between labelled-nutrient uptake and wool growth (Experiment 5).

Results were considered significantly different when $P < 0.05$.

5.3. RESULTS

The ß-counter was unable to separate the counts generated by the ^{35}Sulphur and ^{14}Carbon due to their similar energy levels, thus the radiolabelled nutrient uptake reported

in trial 5a were expressed as total CPM per milligram of tissue. In trial 5c the uptake of both ^{3}H-glucose and ^{14}C-glucose was studied and in Experiment 5, ^{3}H-glucose was used in place of ^{14}C-glucose.

5.3.1. Trial 5a - Media trial

The ANOVA indicated that there was a significant effect of media on the uptake of nutrients (P = 0.001) but there was no strain variation (P = 0.402) nor any interaction (P = 0.821) between media and strain. In both the finewool and strongwool Merino the highest level of uptake was in the two buffered salt solutions (Table 5.1), with uptake being significantly lower in the two commercially-prepared media.

Table 5.1. Total labelled-nutrient uptake (CPM/mg tissue) by the skin and follicles of finewool and strongwool Merinos (mean ± s.e.m) in four different media and the pre-incubation pH (pre-pH) and post-incubation pH (post-pH) of the media

Media	post-pH	Finewool	Strongwool
Krebs-Ringer solution	7.5	1342 ± 75.8 ^{a}	1427 ± 182.0 ^{a}
PBS	6.7	928 ± 42.1 ^{b}	1058 ± 142.8 ^{a}
Media 199	7.2	719 ± 46.0 ^{c}	678 ± 42.6 ^{b}
Williams E	7.6	457 ± 18.0 ^{d}	505 ± 60.9 ^{c}

Different superscripts within strains represent significant differences between means within strains at $P < 0.05$.

5.3.2. Trial 5b - Linearity trial

The uptake of ^{14}C-glucose by the strongwool and finewool Merino followed a linear trend for four hours of incubation (Fig. 5.1) with the correlation coefficient being 0.985 and 0.995 (P < 0.01) for both strains respectively. There was no uptake of labelled-glucose by the boiled blank skin throughout the incubation (Fig. 5.1).

The counts generated with ^{35}S-cystine were very high (> 46166 CPM) and thus lower doses were used in subsequent trials. The uptake of radiolabelled cystine was linear for three hours of incubation (r = 0.993 and 0.991 for strongwool and finewool Merinos, respectively, P < 0.01). The uptake appeared to plateau after three to four hours of incubation in both strains of Merinos (Fig 5.2b). The boiled blank incorporated cystine at a

greater rate than the skin of either the strongwool or finewool Merino (Fig 5.2a) due to the apparent adsorption of cystine to the boiled skin. It was likely that the cystine was binding to the proteins denatured by the boiling of the skin prior to incubation. For this reason, incubation at 4°C was considered a more appropriate blank than boiled skin and was used in subsequent trials (Trial 5c and Experiment 5).

5.3.3. Trial 5c - Site of uptake trial

The skin was incubated in each of the three isotopes separately since the autoradiographic technique is incapable of discerning different isotopes. All of the test skin samples showed high levels of isotope detected by the ß-counter, whereas the blank samples (incubated at 4°C) did not have counts greater than background, with the exception of the cystine blank (Table 5.2). However, the cystine blank was still significantly lower than the samples incubated in the radioisotopes ($P < 0.001$).

Table 5.2. Nutrient uptake of three skin samples from a strongwool (SW) and a finewool (FW) Merino incubated with ^{35}S-cystine, ^{14}C-glucose and ^{3}H-glucose at 37°C and a blank skin sample incubated with the aforementioned isotopes at 4°C (mean CPM/mg tissue ± sem)

	^{35}S-cystine	^{14}C-glucose	^{3}H-glucose
SW	1325 ± 65.8	626 ± 76.8	217 ± 5.8
FW	1560 ± 88.3	540 ± 34.4	82 ± 8.9
Blank	220 ± 23.2	25 ± 4.2	5 ± 0.7

Examination of blank samples indicated that there was no isotope present in the skin or the follicles. Radiolabelled cystine was confined to the follicle region and was located mainly within the keratogenous area of the follicle (Plate 5.1). The samples incubated with radiolabelled glucose, even after 140 days exposure, were identical to the blank samples and the site of glucose uptake could not be identified.

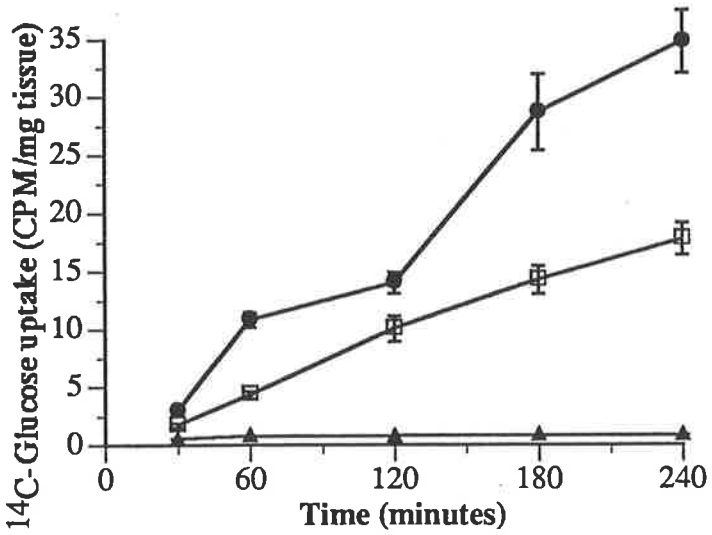


Figure 5.1. The uptake of ^{14}C-glucose over time by the skin of a finewool Merino (□), a stongwool Merino (●) and a blank skin sample (▲) when grown in vitro in Krebs - Ringer buffer, ^{35}S - cystine and ^{14}C-glucose (mean ± sem)

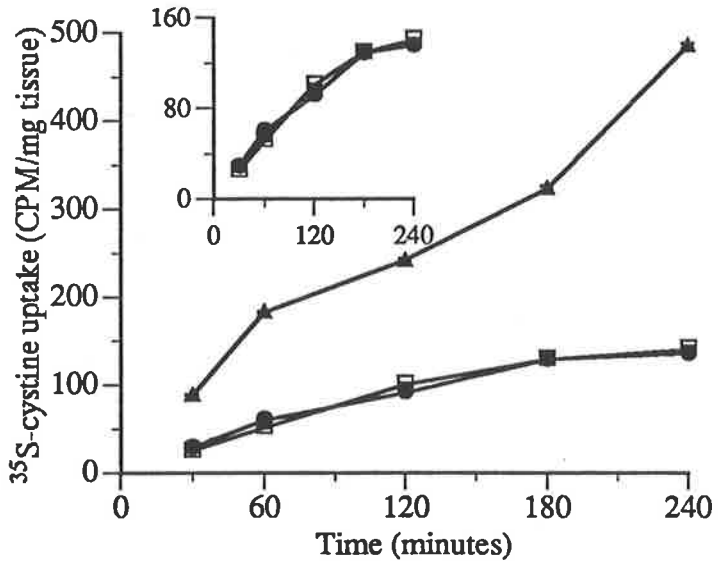


Figure 5.2. The uptake of ^{35}S-cystine over time by the skin of a finewool Merino (□), a stongwool Merino (●) and a blank skin sample (▲), when grown *in vitro* in Krebs - Ringer buffer, glucose and ^{35}S-cystine (mean ± sem)

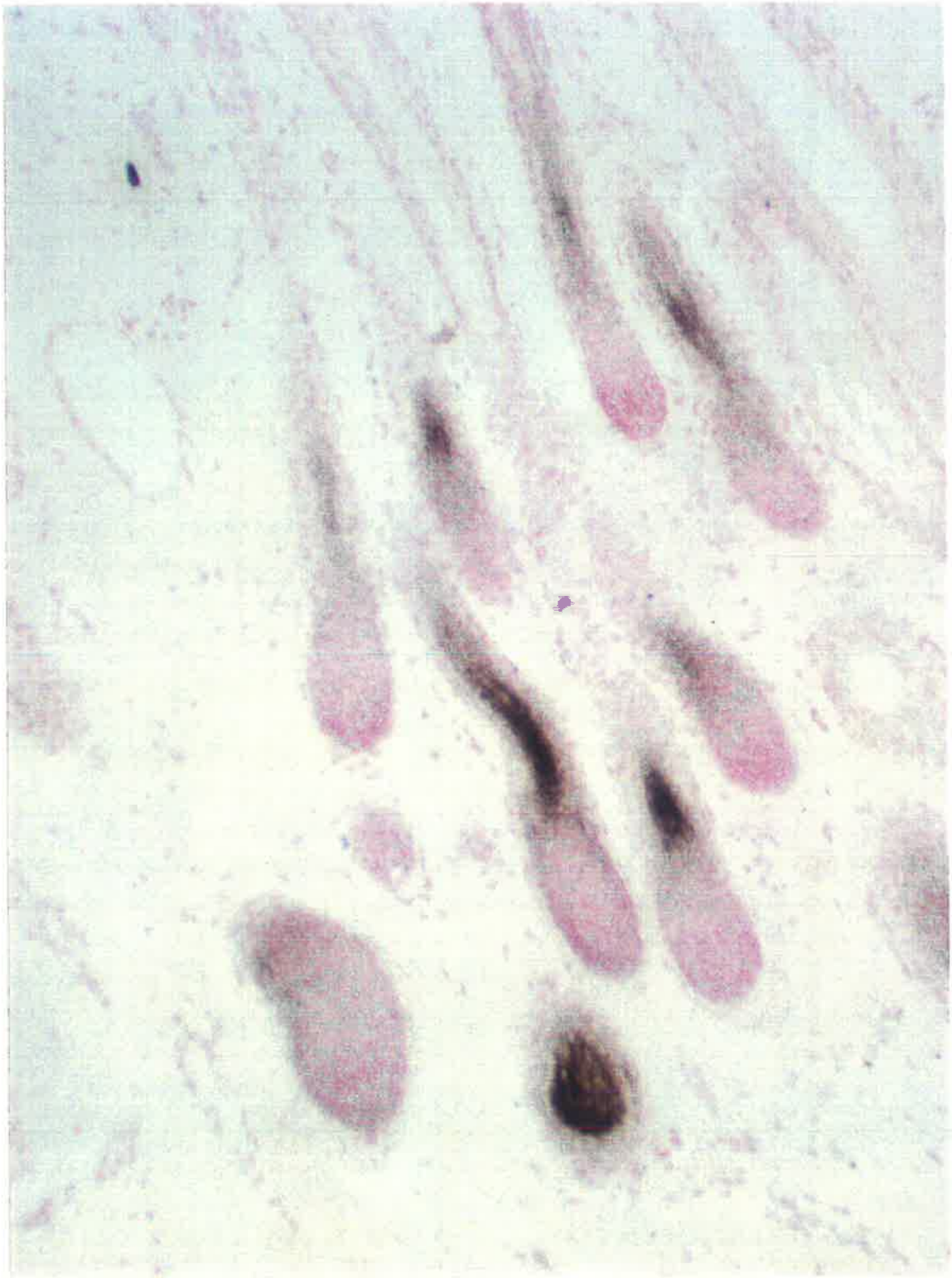


Plate 5.1. Autoradiograph of the uptake of ^{35}S-cystine by the skin and follicles incubated in Krebs-Ringer buffer with ^{35}S-cystine. The only regions with radiolabelled cystine, is in the keratogenous zone of the follicles.

5.3.4. The uptake of ^{3}H-glucose and ^{35}S-cystine by skin and follicles from finewool and strongwool Merinos (Experiment 5)

The sheep consumed all of their rations throughout the stabilisation period, although one of the finewool Merinos (sheep 992) did not eat all of the feed offered during the final four days of the trial (total refusal = 1.5kg). Wool growth per unit area of skin per day differed significantly between the strongwool and finewool Merinos during the final 18 days of the trial (P = 0.017; Table 5.3).

Table 5.3. Wool growth rates (mg/cm^2/d) and the average level of radiolabelled nutrient uptake (CPM/mg tissue) by skin samples taken from four finewool and four strongwool Merinos and incubated in ^{3}H-glucose and ^{35}S-cystine for three hours (Mean ± sem)

	Finewool	Strongwool	P =
Wool growth	0.28 ± 0.031^{a}	0.69 ± 0.103^{b}	0.017
^{3}H-glucose uptake	256 ± 26.2^{a}	387 ± 45.9^{b}	0.049
^{35}S-cystine uptake	115 ± 17.9^{a}	155 ± 24.2^{a}	0.230

The sampling and subsequent incubation occurred on two consecutive days. There was no difference in the uptake of radiolabelled nutrients between the two blanks between the two days (Appendix 5.2), nor was there any significant day effect (P = 0.238) or interaction of day with labelled-nutrient or strain, as indicated by the ANOVA. There was a significant difference in the pH of the media after the three hour incubation period (P = 0.004) between the first (pH = 7.6 ± 0.06) and second day (pH = 7.7 ± 0.05) of the trial.

The level of ^{3}H-glucose uptake per unit weight of tissue was significantly different between the two strains (P = 0.049) but there was no difference in the uptake of ^{35}S-cystine (P = 0.230; Table 5.3) between the skin from the finewool and strongwool Merinos. There was no correlation between wool growth and the uptake of either radiolabelled nutrient (Figures 5.3 and 5.4; P > 0.1; r = 0.35 & r = 0.10 for ^{3}H-glucose and ^{35}S-cystine uptake respectively).

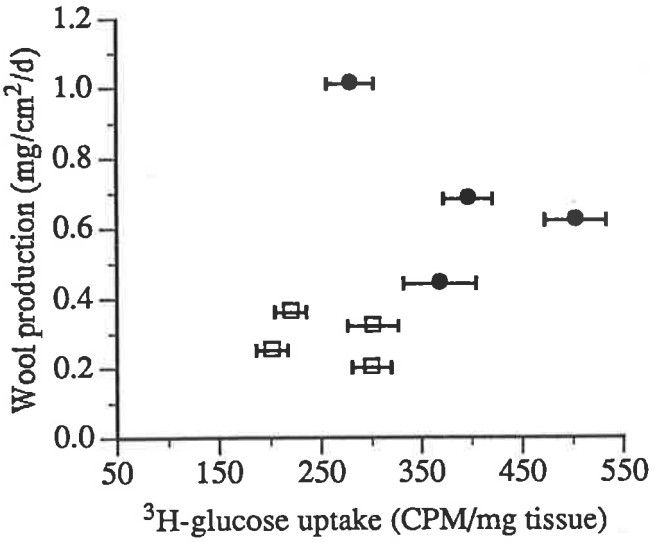


Figure 5.3. Relationship between wool production and the uptake of ^{3}H-glucose by skin from finewool (□) and strongwool Merinos (●) when the skin was incubated in Krebs-Ringer buffer with radiolabelled cystine and glucose (standard error bars are shown) (r = 0.35)

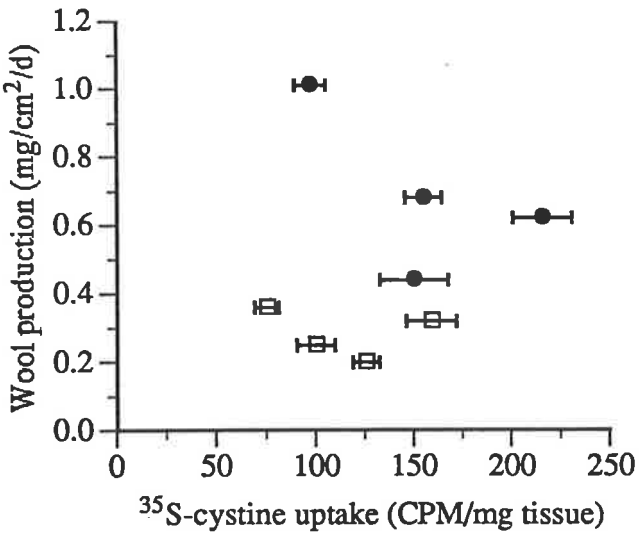


Figure 5.4. Relationship between wool production and the uptake of ^{35}S-cystine by skin from finewool (□) and strongwool Merinos (●) when the skin was incubated in Krebs-Ringer buffer with radiolabelled cystine and glucose (standard error bars are shown) (r = 0.10)

5.4. DISCUSSION

5.4.1. Glucose uptake

The supply of energy to wool follicles is not a major factor controlling wool growth (Reis, 1969; Black *et al.*, 1973; Kempton, 1979; Reis *et al.*, 1988) but it has been observed to influence length growth rate when protein is not limiting (Reis *et al.*, 1988; 1992). Energy is needed to provide ATP for mitosis and protein synthesis and so the present study was initiated to determine if there was a difference in the ability of skin and follicles of two strains of Merinos to incorporate glucose. Cruickshank *et al.* (1957) found that the transfer of glucose across cell membranes in skin from guinea pig ears is actively-controlled. They showed that the rate of glucose utilisation by the skin remained unchanged when incubated over a range of glucose concentrations. When the skin was incubated in other carbohydrates such as fructose, the uptake varied in proportion to the concentration of the sugar in the media, suggesting that fructose passes into the cells by simple diffusion (Cruickshank *et al.*, 1957). This ability of the skin to actively transfer glucose from an extracellular pool may be related to wool growth differences. In the present study, there was a significant difference between the finewool and strongwool Merinos in the uptake of ^{3}H-glucose from an extracellular pool. This difference in uptake level may reflect a difference in the ability of the skin and follicles to transfer glucose across follicle cell membranes. However, the difference was more likely to represent a simple allometric effect, whereby the presence of a greater germinative volume and cell production rate in the strongwool Merinos (Chapter 2) resulted in more glucose being required for follicle metabolism. Ultimately, there would be similar levels of uptake of glucose per individual cell between the two strains, however since there are more cells in the high-wool producers the total amount of glucose being absorbed would need to be greater in the strongwool than the finewool Merinos.

The regions where labelled glucose was situated within the skin and follicles were not identified in Trial 5c when the skin strips were incubated with either ^{3}H-glucose or ^{14}C-glucose and the reason for this can only be speculated upon. There were very high counts generated per milligram of tissue for both isotopes so there was definitely glucose in the samples before tissue processing. This suggests that the label may have been removed

after the TCIA washing and before exposure to the radiographic film. For example, with the use of 6-^{3}H-glucose, the glucose may have been oxidised prior to ß-counting, thus resulting in the ^{3}H being present as ^{3}H-water. In the dehydration step of tissue processing the labelled water would have been removed, thus there would be no isotope present in the skin samples when exposed to the radiographic film. Alternatively, the fate of the radiolabelled carbon in ^{14}C-glucose is either as $^{14}CO_2$ or lactate. If it was the former, the gas would be released rapidly into the extracellular pool and there would not have been any counts generated from the sample, although this was not the case in the current work as counts were present in the tissue. Finally, the labelled glucose may have been generally distributed throughout the skin, unlike the cystine which is concentrated within the fibre, and thus the aggregations of silver grains from the ^{3}H or ^{14}C were not visible above background.

In vitro studies by others have indicated that hair follicles in the rat and human preferentially metabolise glucose to lactate, rather than to CO_2 by aerobic glycolysis (Adachi & Uno, 1968; Kealey *et al.*, 1991; Philpott & Kealey, 1991). Leng and Stephenson (1965) found that both lactate and CO_2 were produced in the wool follicle, but no indication was given as to the preferred glycolytic pathway. These reports suggest that it should be possible to detect glucose or glucose breakdown products *in situ*. In fact, Ryder (1958) was able to detect labelled glucose in the follicles of mice injected with ^{14}C-glucose. His autoradiographs indicated radioactivity in the follicle bulb, lower parts of the fibre and in the outer root sheath with the amount of activity in each region dependant on the time after injection. Ryder suggested that the radioactivity in the bulb was likely to be radiolabelled glucose or a secondary compound such as labelled glucose-1 or 6-phosphate deposited directly from the blood stream as an energy source for mitosis. The radioactivity in the outer root sheath was only associated with the presence of glycogen which took more than eight hours to be deposited. Thus, in the present study it would be unlikely to find activity in the outer root sheath after only three hours incubation. Rather, it should have been possible to detect activity in the follicle bulb and lower regions of the keratogenous zones. Ryder made no mention that there was any activity in the dermis in the epidermis,

thus it can be tentatively concluded that the activity associated with glucose uptake in the present study was due to uptake by the follicles.

5.4.2. Cystine uptake

^{35}S-Cystine was mainly incorporated into the follicle cells situated in the keratogenous region with lesser amounts being taken up by the bulb cells (Plate 5.2) in agreement with studies by Downes *et al.* (1962). It is concluded that the activity associated with cystine uptake was due to active uptake of the amino acid by the follicles for protein synthesis.

It is generally accepted that increasing the amounts of undegraded sulphur amino-acids available to the sheep (i.e. by providing cystine/methionine systemically, parenterally or via the abomasum or duodenum) will increase wool production in Merino sheep (Reis, 1967; Dryden *et al.*, 1969; Downes *et al.*, 1970; Langlands, 1970; Robards, 1971; Bird & Moir, 1972; Williams *et al.*, 1972a, b; Barger *et al.*, 1973; Reis *et al.*, 1973; Dove & Robards, 1974; Ferguson, 1975). It has been further suggested that differences in wool growth between sheep may be due to differences in the ability of the follicles to incorporate nutrients (Black, 1987). In particular, sheep genetically-superior in wool production have a greater response to sulphur amino-acid supplements than low-wool producers (Williams *et al.*, 1972b) although this may simply reflect a lower sulphur amino-acid requirement, causing another nutrient to become limiting in the latter group of sheep. The incorporation of the major limiting amino acid, cystine, was examined in the present *in vitro* study. No difference was found in the levels of uptake for ^{35}S-cystine between the two strains of Merino, nor was there any correlation with wool growth. This indicates that there is no difference between follicles from different sheep in their ability to incorporate ^{35}S-cystine into the follicle from an extracellular pool. Thus it can tentatively be concluded that wool growth is not limited by the ability of the follicle to obtain cystine when there is an excess of the nutrient in the extracellular pool. Rather, it can be speculated that wool growth is limited by the supply of cystine to the skin and follicles. If the same reasoning as that used to explain the difference in glucose uptake is applied to the present situation, then there must be less cystine incorporated per cell in the strongwool Merino than in the finewool Merino in order to get similar total levels of ^{35}S-cystine uptake between the two strains.

This theory is supported by the *in vivo* observation that there is less cystine present in the fibres of high-wool producers than low-wool producers when cystine content is expressed as a percent of total fibre output (Reis *et al.*, 1967; Williams *et al.*, 1972b; Williams & Winston, 1987; Reis *et al.*, 1989).

5.4.3. The *in vitro* technique

The uptake of radiolabelled glucose and cystine by skin strips from two strains of Merinos was examined using *in vitro* techniques in order to strictly control the amount of nutrient available to the skin and follicle and to remove any general metabolic effect, e.g. endocrine status. The skin culture technique was successful in that both radiolabelled glucose and cystine were being actively-absorbed from the medium when compared with the low levels of nutrient present in the 4°C blank samples (Table 5.2).

The Krebs-Ringer solution provided the greatest rates of growth compared to the commercially-prepared media during the four hour incubation period. Various media have been used for the culture of skin strips, for example Krebs-Ringer (Cruickshank *et al.*, 1957), Waymouths media MB752 (Ward & Harris, 1976), Tyrode or modified Hams media supplemented with foetal calf serum (Frater & Whitmore, 1973) and TC199 (Takashima *et al.*, 1970). Krebs-Ringer (Leng & Stephenson, 1965; Adachi & Uno, 1968) and Williams E (Philpott *et al.*, 1989, 1990; Philpott & Kealey, 1991) also have been successfully used for the culture of isolated follicles. In the present trial, the Krebs-Ringer solution provided the best growth conditions when expressed as total radioactivity per unit weight of tissue, possibly due to its superior buffering capacity. In trial 5a, it was the only medium in which the pH did not change during the incubation period (Table 5.1). However, Krebs-Ringer buffer is unlikely to be adequate as a long-term medium, as observed by others (Hynd, person. comm.) and the media may have contributed to the plateau observed in the uptake of ^{35}S-cystine in Trial 5b. The decrease in labelled-nutrient uptake after three hours may have been due to the absence of nutrients in the media and the subsequent depletion of nutrient reserves in the skin. These reserves apparently were adequate to maintain a linear incorporation of ^{35}S-cystine into the fibre cells for the first three hours. Cruickshank *et al.* (1957) found that the respiration rate of guinea pig ears incubated in Krebs-Ringer buffer decreased over a 24 hour period and the addition of glucose, fructose, lactate or pyruvate (<

0.02mM) stimulated respiration rate to a level which was maintained for 24 hours. This suggests that Krebs-Ringer buffer with glucose (0.67μM) and cystine (17.5μM) is insufficient to keep skin alive for any extended period. Alternatively the plateau may have resulted from insufficient nutrients being able to move across the slices of skin. This is further supported by the fact that nobody has been able to adequately grow adult fibre and skin pieces in culture for extended periods of time, although there are a number of reports of growth of embryonic skin pieces (Hardy, 1949; Hardy & Lyne, 1956b; Kollar, 1966; Bartosova *et al.*, 1971; Frater & Whitmore, 1973; Frater, 1980). Foetal skin is more conducive to growth *in vitro* due to the apparent greater ability of juvenile follicles to aggregate or repair damage. Whether the growth is a function of the follicles or the embryo itself is unknown since an embryonic digest is necessary to maintain growth of 3-5 day old skin (Frater and Whitmore, 1973), although these workers only assessed growth subjectively. Frater and Whitmore (1973) also commented that smaller dermal pieces containing fewer follicles grew faster and for a longer period of time than did larger pieces of 3-5 day old mice skin. They suggested that this lack of growth may have been due to diffusion limitations.

To further understand the metabolism of the follicle and its uptake mechanisms, it may be necessary to use isolated follicles in culture as described by Philpott and Kealey (1991) and recently developed for wool follicles (Hynd *et al.*, 1992).

5.4.4. Conclusions

The hypothesis that differences in wool growth between sheep in similar environments are due to differences in the incorporation of glucose was supported since there was a significant difference between ^{3}H-glucose uptake by the skin of finewool and strongwool Merinos. It is speculated that this is purely an allometric effect resulting from a greater germinative volume in the strongwool Merinos. There also was no difference in ^{35}S-cystine uptake between strains of Merinos. Thus, the hypothesis concerning the uptake of ^{35}S-cystine was not supported. The uptake of cystine and glucose, their interplay with blood flow to the skin and plasma concentrations and their relationship with wool growth, volume of germinative tissue in the skin and strain of Merino need to be further examined *in vivo*, and a preliminary study of this is reported in the following chapter.

CHAPTER 6.

PRELIMINARY *IN VIVO* STUDY OF CYSTINE AND GLUCOSE UPTAKE FROM THE PLASMA BY THE SKIN AND FOLLICLES OF FINEWOOL AND STRONGWOOL MERINOS

" ' How long ?' asked Durf again.

' I don't know!' the poor little scientist stressed yet again. Durf was being like the usual idiots, the people who acted as if science was a series of definite facts and scientists benign, all-seeing, all-knowing teachers. The truth was that every dorr that science opened revealed a corridoor full of them, all barred and bolted." (Elton, 1989)

CHAPTER 6. PRELIMINARY *IN VIVO* STUDY OF ^{35}S-CYSTINE AND ^{3}H-GLUCOSE INCORPORATION BY THE SKIN AND FOLLICLES FROM THE PLASMA OF FINEWOOL AND STRONGWOOL MERINOS

6.1. INTRODUCTION

In Chapter 5 it was shown that, *in vitro*, there was no difference in the level of ^{35}S-cystine uptake from an extracellular pool by the skin of strongwool and finewool Merinos. On initial consideration, this finding is not unexpected since the irreversible loss rate of cystine from the plasma is not greater in high-wool producers compared to low-wool producers (Fleece-plus *vs* Fleece-minus Merinos - Williams *et al.*, 1972a; Williams 1976; Williams & Thornberry, 1991; Fleeceweight selected *vs* control Romneys - Lee & Harris, 1991). It is important to note that these data were calculated from whole body infusions of ^{35}S-cystine, either over a period of time or as a single injection, and the subsequent rates of removal and irreversible loss rates of ^{35}S-cystine from the plasma into the extracellular space were estimated. However, the uptake of cystine by the skin and follicles *per se*, cannot be estimated from such whole body clearance rates since it is unknown whether there is a differential partitioning of the blood between the skin and other organs. A further complication is that there is a difference in the rate of blood flow to the skin of finewool and strongwool Merinos (Chapter 3). Another confounding factor involved in the uptake of cystine is the actual concentration of cystine in the plasma. It has been shown that there is a consistently lower concentration of cystine in the venous plasma of the Fleece-plus sheep than the venous plasma of the Fleece-minus sheep (Williams *et al.*, 1972a,b; 1986; Williams, 1976; 1984; Hough *et al.*, 1988). This implies that there may be a relationship between the concentration of cystine in plasma and wool growth. Similar observations have not been reported in other flocks, nor has the cystine concentration of arterial plasma been examined. The present chapter will attempt to address some of these relationships.

In vitro, there is a difference in the level of ^{3}H-glucose uptake between strains of Merinos (Chapter 5). These data suggest that ^{3}H-glucose uptake by the skin and follicles may have been related to the amount of germinative tissue present in the skin, although this

was not examined. The utilisation of glucose by the skin *in vivo* has not been examined in Merinos, but it is known that glucose is used by the skin as an energy source in Romney sheep (Harris *et al.*, 1989).

The work presented in this chapter examines the following hypotheses for both cystine and glucose:

1. That radiolabelled nutrient uptake from the plasma by the skin and follicles is related to the amount of nutrient supplied to the skin;
2. That radiolabelled nutrient uptake from the plasma by the skin and follicles is related to wool growth;
3. That radiolabelled nutrient uptake from the plasma by the skin and follicles does differ between finewool and strongwool Merinos; and
4. That radiolabelled nutrient uptake from the plasma by the skin and follicles is related to the volume of germinative tissue in the skin.

There are two methods for determining the uptake of nutrients from an extracellular space. Both techniques involve the addition of identifiable compounds (eg radio- or immuno-labelled compounds) into the body or region of interest. The clearance or irreversible loss rate is estimated by determining the rate of removal of the compound from the system. Alternatively, uptake can be measured by examining the concentration of the nutrient in the organ of interest. Both of these methods were investigated prior to the testing of the above hypotheses.

A surgical preparation has been developed (McDonald *et al.*, 1982; Zoltie *et al.*, 1988; Harris *et al.*, 1988) which allows the study of an 'isolated patch of skin'. The procedure enables measurements of uptake and output of metabolites in a defined area of skin over the caudal flank and cranial thigh regions by the placement of catheters in the deep circumflex iliac artery and vein. This method has been used to study blood flow through the skin (Dellow *et al.*, 1988; Harris *et al.*, 1989) and the protein and energy metabolism of the skin of sheep by estimating the arterio-venous differences of various metabolites (Harris *et al.*, 1989). The present work examines the usefulness of this technique for studying the uptake of labelled-nutrients from the plasma by the skin and follicles.

6.2. MATERIALS AND METHODS

6.2.1. General

6.2.1.1. Materials

Ultrafiltration centrifuge tubes (Ultrafree-MC) which contained 10 000 NMWL Low-Protein-Binding Regenerated Cellulose ultrafiltration membranes, and the mini-filter units (Millex-HV4) which had a pore size of 0.45μm and a 4mm diameter filter were obtained from Millipore (Bedford, Massachusetts). The HPLC analyses was undertaken with a Pico-Tag® column for free amino acid analysis (30cm) containing Dimethyloctadecylsilyl bonded amorphous silica (Waters, Millipore Corporation; Milford, Massachusetts). Triethylamine (Sequanal grade), phenylisothiocyanate (PITC; Sequanal grade) and amino acid standard Amino acid standard H) were products of Pierce (Rockford, Illinois). Microvials were obtained from Adelab (Microsun Inserts made in Germany; Adelaide, SA).

Methionine sulphone, peroxidase (POD), glucose oxidase (GOD) and 2,2'-Azino-di-(3-ethylbenzthiazoline)-6-sulphonate (ABTS) were produced by Sigma Chemical Co. (St Louis, Missouri).

Methanol (HPLC grade), acetonitrile-190 (HPLC grade) and α-D+-glucose were supplied by AJAX Chemicals (Auburn, NSW). Sodium acetate and sulphosalacylic acid (SSA) were obtained from May and Baker, Australia Ltd (West Footscray, Vic.). Perchloric acid ($HClO_4$; sp. gr. 1.70), trichloroacetic acid (TClA; AnalaR), sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), disodium hydrogen phosphate (Na_2HPO_4) and magnesium sulphate ($MgSO_4.H_2O$) were products of BDH Chemicals (Kilsyth, NSW).

L-^{35}S-cysteine hydrochloride (114mCi/mmol), D-[6-^{3}H]-glucose (23Ci/mmol) and scintillant (ACSII) were obtained from Amersham Australia (North Ryde, NSW) and Soluene 350 was from Packard Instruments (Downers Grove, Illinois).

Saline and heparin sodium [5000U/ml] were products of Commonwealth Serum Laboratories (Melbourne, Vic.) and the heparinised plasma tubes with Lithium Heparin (125 I.U./tube) were supplied by Disposable Products (Adelaide, SA).

Evans blue was a product from Gurrs (London, England). Disposable cuvettes were composed of optical polystyrene and obtained from Lab Supply (Adelaide, SA.) and the clear vinyl tubing was made by Dural Plastics and Engineering (Dural, Australia).

6.2.1.2. Sheep

The sheep were housed in metabolism crates in a climate-control room (Temperature - 18°C; Light between 0830 and 1930h), fed lucerne and grain-based pellets *ad libitum* (Milling Industries; Murray Bridge, SA) and had free access to water.

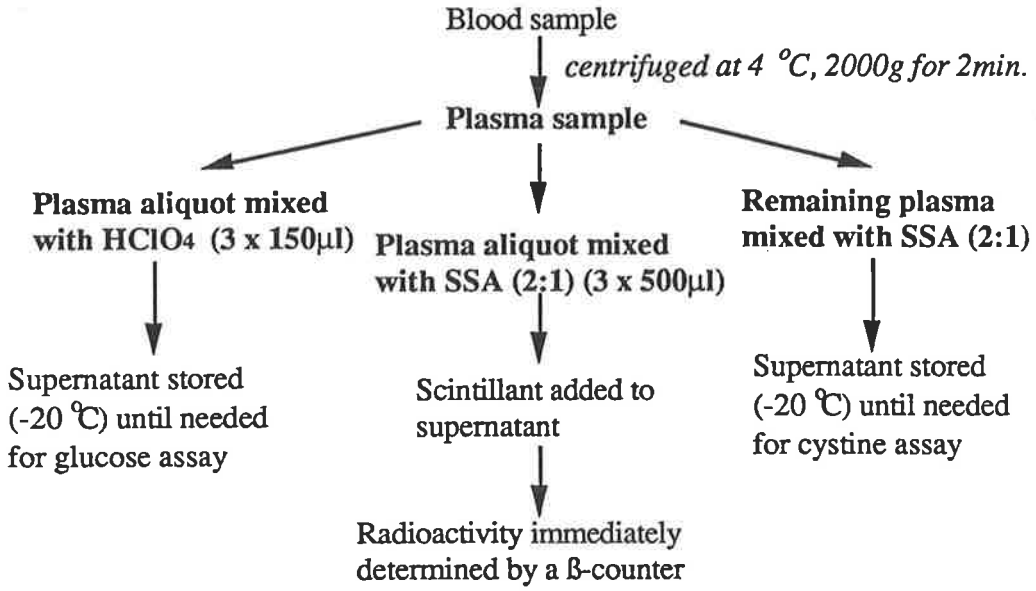
6.2.1.3. Skin sampling and analysis

Two skin biopsies were taken as described in Section 2.2.3 immediately prior to blood sampling. The skin samples were cut in half and placed in TClA (5% w/v) for 15mins (x four times), trimmed of excess fat and wool, weighed, placed in scintillation vials, and then solubilised in Soluene 350 (1.0ml) and shaken overnight in a waterbath (60°C). After cooling, HCl (1M; 1.25ml) and scintillant (5ml) were added to the vials which were stored in the dark for 24 to 48 hours to decrease chemoluminescence. The samples were then placed in a ß-counter (LKB Wallac Beta counter) and the counts per minute (CPM) were recorded.

6.2.1.4. Blood sampling and analysis

Blood samples were withdrawn from the animals via a catheter in the deep circumflex iliac artery using a hand-held syringe and the blood was immediately transferred to plasma tubes with heparin on ice and centrifuged within 15 minutes of sampling (2000 *g*; 5 min.; 4°C). The plasma was subsequently sub-divided as shown in Figure 6.1. Aliquots of plasma (150µl) were taken and these samples were deproteinised with $HClO_4$ (1:1; 0.6M), centrifuged (2000 *g*; 2min. at 4°C) and the supernatant was decanted into an eppendorf tube and stored at -20°C until the glucose assay was performed. The remaining plasma was deproteinised with SSA (20% w/v) in a ratio of 2 parts plasma to one part SSA. All plasma was deproteinised within 30 minutes after sampling. After centrifugation (2000 *g*; 10 min.; 4°C), aliquots (3 x 0.5ml) of the supernatant were placed in plastic scintillation vials. Scintillant (2.5ml) was added to the vials with the aliquots and were stored in the dark for 12 hours to decrease chemoluminescence. The deproteinised plasma samples were then placed in a ß-counter (LKB Wallac Beta counter) and the counts per minute (CPM) were recorded. The remaining plasma was frozen in liquid N_2 and stored at -20°C until further analysis was possible.

Figure 6.1. The distribution and handling protocol for arterial and venous blood samples collected from the sheep for the determination of the radioactivity, glucose and cystine concentration of deproteinised plasma samples



6.2.1.5. Isolated cutaneous patch

The sheep underwent bilateral surgery for the placement of catheters in the circumflex iliac artery and vein in both the left and right abdominal flank, as described in Section 4.2.2. Both vessels were catheterised with clear vinyl tubing (i.d. 0.50mm, o.d. 0.80mm) in a retrograde direction. The blood flow through the artery was controlled with silk thread and a cannula (18G) was inserted into the artery through which a catheter was placed in the vessel. The venous wall was snipped with microscissors and the wall was lifted with a small piece of wire to allow entry of the catheter into the vessel. The catheters were gently tied in the vessels to prohibit movement of the catheter but not hinder blood flow past the catheter. The catheters were also tied to the tensor fasciae latae muscle with silk and a drop of super glue was then placed on each silk to stabilise the catheter. Disturbance to the circumflex iliac artery and vein was minimal since very little of the connective tissue was cleared from the vessels and surrounding area. The catheters were exteriorised by passing them subcutaneously along the medial surface of the thigh to exit in the midthigh region. The catheters were cleared of blood and maintained with a constant infusion of heparinised saline (230iu/ml at 0.15ml/min) provided by a continuous flow pump (Minipuls 2; Gilson, Villiers, France).

6.2.1.6. Infusate

L-^{35}S-cysteine hydrochloride (114mCi/mmol) and D-[6-^{3}H] glucose (23Ci/mmol) were mixed with sterile heparinised saline (20U/ml) to the activity required for each trial and then infused into the sheep via the catheter with the continuous flow pump.

6.2.1.7. Chemical analyses

a) Analysis of cystine

The cystine concentration of the deproteinised plasma was determined by high performance liquid chromatography (HPLC) with the Pico-Tag® method (Cohen *et al.*, 1988). Three standards (Amino acid standard H, plasma amino acids, and cystine alone) and a water blank were used to identify and quantify the peaks. Methionine sulphone was present as an internal standard in each of these standards.

In preparation for chromatography, each deproteinised plasma sample was thawed and mixed (1:1) with internal standard (0.4mM methionine sulphone), loaded into ultrafiltration centrifuge tubes and centrifuged for 30 minutes (2000*g*). An aliquot of each filtrate (50µl) was placed into separate Microsun Inserts and the samples vacuum dried for 2 hours. The samples were then suspended with the redry solution (10µl; 2:1:1 mix of methanol, sodium acetate (1M), triethylamine), mixed, redried on a vacuum drier (2h) and stored dessicated overnight (-20°C). The following morning the samples were resuspended in derivatising solution (20µl; 7:1:1:1 mix of methanol, water, Triethylamine, PITC), mixed and allowed to stand at room temperature for 10 minutes. The samples were then dried on a vacuum drier (2h) after which they were suspended in 100µl of sample diluent (5M Na_2HPO_4, pH7.4; 5%v/v acetonitrile), mixed and then filtered with a mini-filter unit into new microvials and loaded onto an auto-sampler.

The samples and standards were analysed on a Waters Maxima 820 Chromatography Workstation using a Pico-Tag® column, a flow rate of 1ml/min, a column temperature of 46°C and detection at 254nm (Waters Model 441 Absorbance Detector). The mobile phases of sodium acetate (70mM) with 2% (w/w) acetonitrile (A) and a mixture of acetonitrile (353.7g), nano-pure water (400g) and methanol (118.65g) (B) were pumped in the gradient outlined in Table 6.1. Under these condition, cystine was detected approximately 49 minutes after sample injection.

Table 6.1. The gradient and compositional changes of the mobile phases A* and B† used to elute PICT-derivatised cystine from deproteinised plasma

Time (minutes)	Composition (%)		Gradient§
	Eluent A*	Eluent B†	
0	100	0	-
13.5	97	3	11
24.0	94	6	8
30.0	91	9	5
50.0	64	36	6
60.0	64	36	6
60.5	0	100	6
68.5	0	100	6
69.0	100	0	6
95.0	100	0	Column equilibriation

* Eluent A was a mixture of sodium acetate (70mM) and 2% (w/w) acetonitrile
† Eluent B was a mixture of acetonitrile (353.7g), nano-pure water (400g) and methanol (118.65g)
§ Gradient between compositional change from A to B. The manner in which the gradient steps are connected is described by a curve whereby the degree of convexness is < 6, the degree of concaveness is > 6 and linearity = 6.

The concentration of cystine in deproteinised plasma was estimated as cystine since initial trials indicated that 95% of cysteine was converted to cystine during sample processing. Cystine concentration was expressed in µg/ml of deproteinised plasma and dilution factors were taken into account during the calculations.

b) Analysis of glucose

The glucose concentration of the plasma was assayed enzymatically with glucose oxidase (GOD) and peroxidase (POD) as described by Bergmeyer and Bernt (1974). Components of the enzyme reagent were prepared (Table 6.2) and stored at 4°C for up to 6 weeks.

Triplicate assays were performed for each sample by mixing glucose reagent (1.0ml) with the plasma sample (20µl) in a disposable cuvette. Colour was developed by allowing the sample to stand at room temperature for 30 minutes and then the absorbance was measured at 420nm in a Lambda 5 UV/VIS Spectrophotometer (Bodenseewerk Perkin-Elmer & Co. GmbH, Uberlingen, West Germany). The glucose concentration was determined by

reference to a standard curve prepared with known concentrations of glucose (0 to 1000µg/ml) in $HClO_4$ (0.3M) and water blanks were run with each set of assays to account for background colour of ABTS. Glucose concentration was expressed as µg/ml of deproteinised plasma and the results are the mean ± standard error of the mean for the triplicate assays. Dilution factors were taken into account during the calculations.

Table 6.2. Composition of enzyme reagent* used for glucose assay of plasma deproteinised with $HClO_4$

Component	Amount per 50ml
Phosphate buffer (0.12M) - $NaH_2PO_4.2H_2O$	0.363 g
- Na_2HPO_4	0.612 g
POD (1.5U/ml)	75 U
GOD (9U/ml)	467 U
ABTS (0.92mM)	25 mg

* Made up to 50ml with RO water and adjusted to pH 7.0.

6.3. EXAMINATION OF THE ISOLATED CUTANEOUS PATCH TECHNIQUE

These trials were undertaken to determine the usefulness of the isolated cutaneous patch technique to estimate the clearance rate of nutrients by the skin and follicles.

6.3.1. Experimental procedure

^{35}S-cystine and ^{3}H-glucose were infused into sheep via the catheter placed in the deep circumflex iliac artery (Section 6.2.1.5) at various concentrations for different periods of time as indicated in Table 6.3. Blood was sampled from the venous catheter and skin from the flank region at specific times during the infusion after which the samples were processed and analysed as described in Sections 6.2.1.3 and 6.2.1.4.

6.3.1.1. Trial 6.1.

Blood samples (1-2ml) were taken at 0, 30, 60, 120, 180 and 240 minutes after the start of the infusion and the radioactivity associated with ^{3}H-glucose was detected in the samples as described in Section 6.2.1.4. After the infusion was stopped, the sheep was

slaughtered with a lethal dose of saturated $MgSO_4$, and the cutaneous patch was infused with Evans blue (0.2% w/v).

Table 6.3. Protocol for Trials 6.1 to 6.7 to study the uptake of radiolabelled nutrients by the skin of sheep. The sheep identification (sheep), number of days between surgery and the trial (post surgery), nutrient infused, the total amount of the nutrient infused (infusion), and the length of infusion are indicated

Trial	Sheep	Post surgery (d.)	Nutrient	Infusion	Length (min.)
6.1	1	1	^{3}H-glucose	10μCi	240
6.2	2	1	^{3}H-glucose	50μCi	240
6.3	2	6	^{3}H-glucose	50μCi	120
6.4	2	9	^{35}S-cystine	50μCi	120
6.5	3	1	^{3}H-glucose	50μCi	120
6.6	3	3	^{35}S-cystine	100μCi	120

6.3.1.2. Trial 6.2.

Blood (4-5ml) and skin samples were taken at 0, 30, 60, 90, 120, 180 and 240 minutes after the start of the infusion and the radioactivity associated with ^{3}H-glucose was detected in the samples as described in Sections 6.2.1.3 and 6.2.1.4.

6.3.1.3. Trial 6.3.and 6.4.

Blood (1-2ml) and skin samples were taken at 0, 20, 40, 60, 80, 100 and 120 minutes after the start of the infusion and the radioactivity associated with ^{3}H-glucose and ^{35}S-cystine was detected in the samples as described in Sections 6.2.1.3 and 6.2.1.4.

6.3.1.4. Trial 6.5.

This trial was undertaken to determine if any of the glucose was being removed from the tissue during the TCIA washing. Three skin samples were taken at 0, 30, 60, 90 and 120 minutes after the start of the infusion. Each skin sample was cut in half and one half was weighed and placed directly into Soluene 350 whereas the other half was washed in TCIA prior to placement in Soluene 350. Processing and analysis of both sets of skin where then continued as described in Section 6.2.1.3. All of the TCIA washing solutions were retained

and scintillant (5ml) was added to the TClA prior to the determination of radioactivity with the ß-counter.

6.3.1.5. Trial 6.6.

Skin samples were taken at 0, 30, 60 and 120 minutes after the start of the infusion and analysed for ^{35}S-cystine as described in Section 6.2.1.3.

6.3.1.6. Statistical analysis

The counts obtained from the blood samples were expressed as CPM/ml of plasma and the counts generated from the skin samples were divided by the weights of tissue and expressed as CPM/mg tissue. The mean and standard error of the mean were routinely calculated from the replicates of each period and were presented graphically against time. The coefficient of variation (CV) was estimated from the mean CPM/ml of plasma within infusions to detect the presence of a plateau (Williams *et al.*, 1972a). Samples taken at the start and end of each infusion were compared using the Students t-test to determine if there was a significant increase in the activity of the samples. Results of $P < 0.05$ were considered significant.

6.3.2. Results and Discussion

6.3.2.1. Infusion of ^{3}H-glucose

There was no change in the level of labelled-glucose in the plasma in during the infusion of 2.5µCi/h (Trial 6.1; Fig. 6.2a[inset]). The infusion of ^{3}H-glucose at 12.5µCi/h (Trial 6.2) resulted in a significant increase ($P < 0.001$) in the level of activity over time in the venous plasma samples. In this trial (Trial 6.2), a plateau was reached after 180 minutes of infusion (CV = 11.6%; Fig. 6.2a[inset]) but this level did not provide enough ^{3}H-glucose to cause an increase in activity in the skin (Fig. 6.2b). A plateau in the level of activity in the plasma samples was reached within 40 minutes of infusion when the ^{3}H-glucose was infused at 25µCi per hour (Trial 6.3; CV = 12.0%; Fig. 6.2a) and there was a significant uptake of ^{3}H-glucose by the skin ($P < 0.01$) at this level indicating that a high concentration of label was required to obtain activity in the skin. Despite this, the ^{3}H-glucose content of the skin was extremely low with a maximum of 27CPM being recorded for single samples. This level was below the sensitivity of the ß-counter as counts of less than 30 per minute are not considered to be above background. Activity greater than this

was achieved in the plasma samples but not in the skin, thus it is concluded that using the current method, the levels of ^{3}H-glucose taken up by the skin would be insufficient for valid comparisons between sheep.

It is interesting to consider that when 25μCi were infused per hour (Trial 6.3) there was approximately 4301 CPM/ml of plasma compared to almost undetectable levels in the skin. Trial 6.5 indicated that there was no increase above background levels in the activity present in the TClA washing, thus all the activity present in the skin at sampling remained there until counting. This suggests that very little ^{3}H-glucose was being used by the skin for metabolism or there was a low entry rate of ^{3}H-glucose from the plasma to the extravascular pool, or that there was a very large pool of glucose available for the skin, thus the labelled glucose was only a small proportion of this pool. The latter is likely to be the case, firstly since it is unlikely the movement of ^{3}H-glucosewould be slow and, secondly since Harris *et al.* (1989) showed that glucose is used by the skin as an energy source. They also indicated that the arterial concentration of glucose ranges from 400 to 650 μg/ml. Thus, if blood flow is approximately 4ml/min (Section 5.3.2), there would be at least 1600μg of glucose passing through this region per minute into which only 50μg of radiolabelled glucose was being added per minute. This represents a 1 in 32 dilution effectof labelled glucose to unlabelled glucose. With this amount of dilution, it is not surprising that the level of radioactivity in the skin was almost undetectable.

Harris *et al.* (1989) reported that 11.1% of the glucose passing thorough the cutaneous patch is taken up by the skin area. This implies that there should have been 11% of the infused ^{3}H-glucose present in the skin, in Trial 6.3. After 120 minutes, 50μCi (~2.14×10^{7} CPM) had been infused and, if 11% of this was being taken up by 124g of skin (average weight of skin supplied by the infusion; Harris *et al.*, 1989), there should have been 18.9 CPM/mg of tissue sampled. In the present study, there was only 0.73 CPM/mg of tissue. The reason for this large discrepancy between the observed and expected glucose uptake is unknown but may partially be attributed to the 10 - 20% of blood lost by collateral venous drainage (Dellow *et al.*, 1988) and to the uptake of glucose by the cutaneous trunci. Alternatively, or additionally, the discrepancy between observed and expected CPM may be due to the glucose being metabolised rapidly with no accumulation or storage of any of the resulting ^{3}H-compounds.

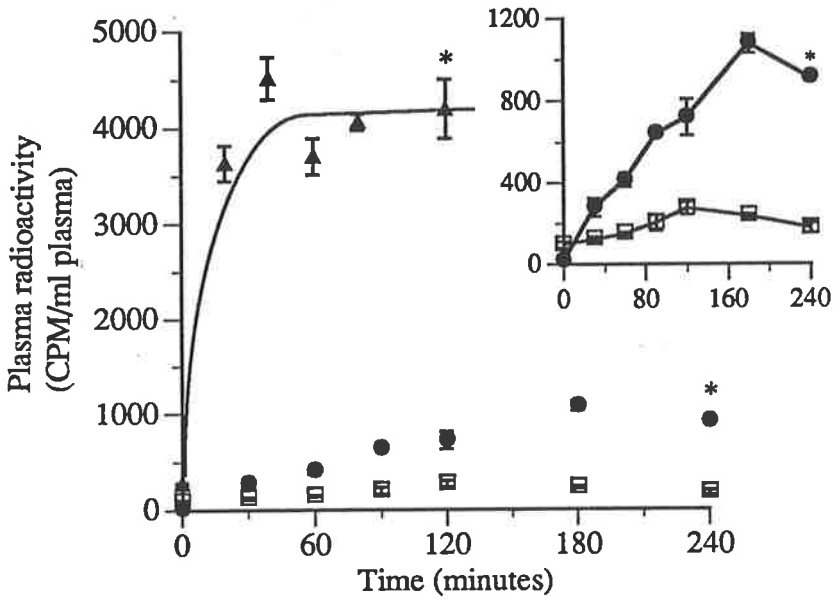


Figure 6.2.a. The activity of plasma sampled from a single sheep during the infusion of ^{3}H-glucose at 2.5μCi/h (Trial 6.1 - □), 12.5μCi/h (Trial 6.2 - ●), and 25μCi/h (Trial 6.3 - ▲) over time.
*** indicates significant difference between 0h and final sample**

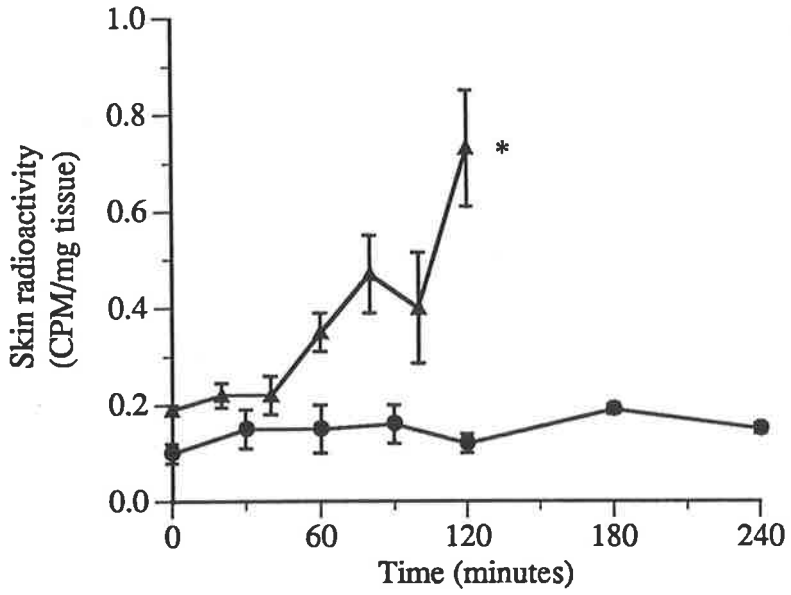


Figure 6.2.b. The activity of skin sampled from a single sheep during the infusion of ^{3}H-glucose a 12.5μCi/h (Trial 6.2 - ●), and 25μCi/h (Trial 6.3 - ▲) over time.
*** indicates significant difference between 0h and final sample**

6.3.2.2. ^{35}S-Cystine infusion

When 25μCi/h of radiolabelled cystine was infused (Trial 6.4.), there was a significant increase in the level of ^{35}S-cystine in the plasma 20 minutes from the start of the infusion ($P < 0.005$), after which the activity in the plasma samples reached a plateau (C.V. = 10.4%; Fig. 6.3a). It was then assumed a plateau was also reached when the cystine was infused at 50μCi/h (Trial 6.6). It was not possible to measure the level of activity in the plasma in the latter trial since the venous catheter was not patent at the time of sampling. The activity of the skin increased significantly when cystine was infused at 25μCi/h (Trial 6.4; $P < 0.005$), however the CPM achieved were not above that required for a detailed analysis of nutrient uptake (Fig. 6.3b). The ^{35}S-cystine content of the skin from Trial 6.6 increased significantly ($P < 0.001$) and was adequate for an analysis of nutrient uptake. The results were extremely erratic (Fig. 6.3b) and there was a large standard error about the mean. This indicates the difficulty in identifying the perfused region. In Figure 6.3b, the depressed 60 minute sample may have been taken from the edge of the perfused area, thus affecting the level of labelled cystine available to the skin. The low level of radioactivity in the skin alternatively may represent the plasma activity as there was a corresponding decrease in activity of the 60 minute plasma sample.

6.3.2.3. Isolated cutaneous patch technique

The arterial catheters remained patent throughout the entire period of each trial, unless there was external interference by the sheep such as chewing or kicking the lines. Such activity resulted in the lines filling with blood and blocking them, or the catheter coming out of the vessel completely. The continued patency of the arterial cannulae permitted the infusions to take place although blood sampling was often restricted by the venous catheters, four out of five being non-patent within 24 hours of surgery (Table 6.4.). This occurred even when the disturbance to the tissue around the lymph node region was kept to a minimum and the catheters were exteriorised above the incision, as suggested by Harris *et al.* (1989).

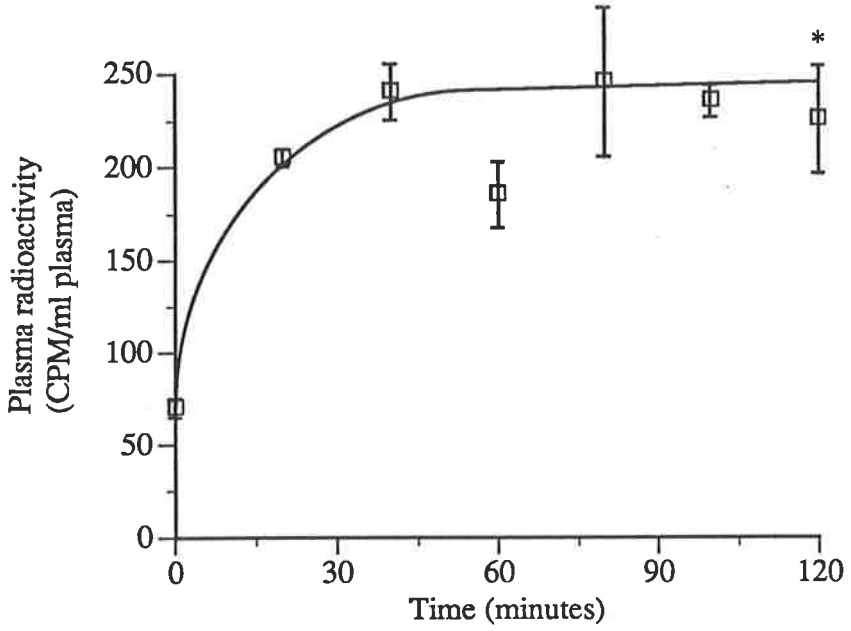


Figure 6.3.a. The activity of plasma sampled from a single sheep during the infusion of ^{35}S-cystine at 25μCi/h (Trial 6.4 - □) over time.
*** indicates significant difference between 0h and final sample**

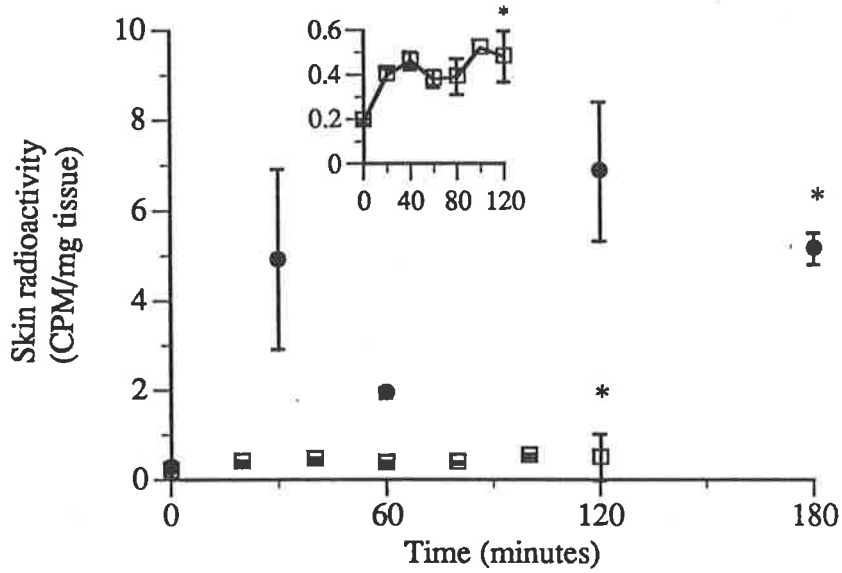


Figure 6.3.b. The activity of plasma sampled from a single sheep during the infusion of ^{35}S-cystine at 25μCi/h (Trial 6.4 - □) and 50μCi/h (Trial 6.6 - ●) over time.
*** indicates significant difference between 0h and final sample**

Table 6.4. Period of patency (hours or days) of the catheters in the deep circumflex iliac vein

Sheep	1	2	3
Patency-nearside	20 h	9 d	24h
Patency-offside	24 h	pulled out *	- #

* This catheter was pulled out of the vein before it became non-patent

\# The offside vein was not cannulated in this sheep

In the present study, it was found that a small incision of the vein wall with microscissors and then using a fine piece of bent wire to lift the wall was the easiest and least traumatic method of inserting the catheter into the vein. Despite this care, there was still early blockage of the venous catheter. The problem may have resulted from leakage of the lymph node, resulting in increased pressure around, and the subsequent collapse of, the vein (Harris *et al.*, 1989).

Evans blue was infused into the artery to identify the perfused area prior to the nutrient infusion, however it was difficult to discern the region due to the short half-life of the solution and the poor visibility of the dye in the skin, in agreement with observations of others (Harris *et al.*, 1989). It was possible to identify that the perfused area was predominantly caudal to the incision whereas the near side was perfused predominantly on the cranial side of the incision. This suggests that the distribution of the nutrient was erratic as observed in Chapter 4 (Fig. 4.1), and it was difficult to determine from where the skin should be sampled.

6.3.3. Conclusion

The systemic infusion of radiolabelled nutrients into sheep to determine the uptake of nutrients from the plasma by the follicles is unlikely to be successful using the proposed isolated cutaneous patch preparation. Although a constant level of activity of substrates can be achieved in the plasma, the uptake of the radiolabelled nutrients by the skin was difficult to quantify due to extremely low levels of activity (particularly with the glucose) and due to the problems associated with identifying the perfused region. Also, the doses required to achieve adequate levels of radioactivity in the skin were almost as high as those used in whole body infusions (e.g. Williams *et al.*, 1972a). Finally, I was unable to maintain the

patency of the venous catheters to allow the sheep to recover from surgery before the experiments were performed. This may have had a confounding effect on the results. It is concluded that the isolated cutaneous patch was not a suitable technique for the present study.

To determine the entry/clearance rate of the nutrient, it is necessary to be able to measure the radioactivity of the plasma by taking samples from a blood vessel of a healthy animal (e.g. Williams *et al.*, 1972a). This was almost impossible in the current study, due to

a) the difficulty in maintaining the patency of the venous catheter for a period long enough for the animals to recover from surgery and thus be 'healthy', and

b) the blood supply to the skin is not completely isolated using this technique since the vessels supply the subcutaneous muscle as well as the skin. Any conclusions made using this preparation concerning the clearance rate of nutrients by the skin are confounded by uptake by the muscle tissue. There is a small amount of both collateral venous drainage and arterial supply within the patch (Dellow *et al.*, 1988) and, although this is considered to be minor and the preparation is adequate for the study of blood flow and metabolism of the skin, it is not possible to conclude that the amount of labelled-nutrient removed is solely due to skin uptake.

The combination of the skin not being completely isolated, the difficulty in defining the perfused area and the high doses required to achieve reasonable levels of isotope in the skin make the isolated cutaneous patch technique unsuitable for the present study. In the following experiment, whole body infusions were used to estimate the uptake of ^{35}S-cystine and ^{3}H-glucose by the skin.

6.4. RADIOLABELLED NUTRIENT UPTAKE

This experiment was undertaken to test the hypotheses presented in Section 6.1.

6.4.1. Experimental procedure

Four finewool and four strongwool Merinos were housed indoors in individual pens for a stabilisation period of 83 days and were fed grain-based pellets (1kg/d; Milling Industries, Murray Bridge, SA). Water was available *ad libitum.* During the stabilisation period, clean wool growth per unit area of skin was measured as described in Section 2.2.3. with the measurement made over the last 14 days of the stabilisation period being used for analysis in this study. The sheep were weighed on the final day of the stabilisation period (Section 2.2.2).

In order to collect arterial blood samples during the infusion, the sheep underwent aseptic surgery to cannulate the deep circumflex iliac artery as described in Section 6.2.1.5. After surgery, the sheep were immediately placed in individual metabolism crates in a climate-control room (22°C; light during 0830 to 1930). The catheters were connected to a continuous flow pump through which heparinised saline was infused to maintain catheter patency as described in Section 6.2.1.5. The animals were fed lucerne chaff (100g) and grain-based pellets (300g) thrice daily (0830h, 1300h, 1730h). Water was available *ad libitum.*

On days five and six after surgery, blood flow through the midside patch was measured with laser Doppler velocimetry (LDV) as described in Section 3.2.2.1 to familiarise the sheep to the machine and to handling. Seven days after surgery, ^{35}S-cystine (1.25μCi/ml) and ^{3}H-glucose (1.25μCi/ml) were infused into the sheep at a rate of 20ml/h for four hours via a catheter which had been placed in the jugular vein on the previous day. Two skin biopsies were taken before the infusion started and were placed in buffered formalin for the determination of the volume of germinative tissue in the skin as described in Section 2.2.4. Prior to the start of the infusion and after four hours of infusion, blood flow through the skin was measured at two sites using LDV (Section 3.2.2.1) and the skin from these sites was taken as described in Section 6.2.1.3. Blood samples (20ml) were also taken from the jugular catheter and the iliac artery catheter (5ml) before the infusion started.

After the infusion was stopped, the animals were removed from the crates and the sheep were anaesthetised with sodium pentobarbitone (i.v. 10 - 15ml; Nembutal®; Boehringer Ingelheim, Artarmon, NSW) via the jugular catheter. The jugular catheter was flushed and a blood sample (20ml) was withdrawn. The carotid artery was then exposed and an arterial blood sample (20ml) was taken from this vessel within two minutes of the animal being disconnected from the infusion line. The animal was then slaughtered by administering a lethal dose of sodium pentobarbitone through the jugular catheter.

Blood and skin samples were processed and the activity of both were determined as described in Sections 6.2.1.3 and 6.2.1.4. Cystine and glucose concentrations of the plasma were determined as described in Section 6.2.1.5. Total germinative volume of the skin was determined as described in Section 2.2.3.2.

6.4.1.6. Calculations, assumptions and statistical analysis

Nutrient flow was calculated from the product of average blood flow through the skin and the concentration of nutrient per unit volume of blood.

Initial trials indicated that there was no ^{3}H overlap into the ^{35}S channel but there was a 5% overlap of counts from the ^{35}S channel into the tritium channel of the β-counter. This overlap was accounted for when the radioactivity of the samples was estimated.

Oxidation of cystine in the plasma is similar between sheep with different wool growth potential under different environmental conditions (Sun *et al.*, 1994), thus in the current trial it was assumed that there was a similar level of oxidation between sheep which accounted for 45% of the radioactivity in the deproteinised plasma (Lee *et al.*, 1993). Thus, the specific radioactivity (SRA) of cystine in the plasma was estimated by Equation 6.1 after the ^{35}S radioactivity associated with the the oxidation products was taken into account. The amount of ^{35}S associated with cystine flowing through the skin was then estimated from the product of the SRA and blood flow.

Equation 6.1.

$$\text{Specific radioactivity of cystine} = \frac{S^{35}\text{ activity in the plasma (CPM / ml)}}{\text{Quantity of cystine in the plasma (ug / ml)}}.$$

It was assumed that radioactivity detected in the skin was primarily associated with cystine in the "bound pool" of the skin. This is justified by the following calculations. For

example, assume the concentration of free cysteine in the skin is 180nmol/g tissue, i.e. 20ug/g tissue, (Lee *et al.*, 1993) and has a SRA of 20% of blood (Lee *et al.*, 1993) and also assume that skin contains approximately 9mg protein / g tissue (given that 3% of the skin is protein - 30% wet weight). Thus, with the fractional synthesis rate of protein being approximately 10% per day (Harris & Lobley, 1991) which is equivalent to 1.7% over 4 hours, then 150ug of protein is synthesised over the 4 hour infusion period. This indicates that there is likely to be a much greater percentage of radioactivity in the bound pool than in the free cystine pool. This assumption is also supported by autoradiographic results which indicated that the majority of the activity was associated with the keratogenous zone of the follicle (Section 5.3.3.).

The amount of isotope retained in the skin (CPM/unit weight of tissue) was directly compared with the concentration of isotope flowing through the skin (CPM/min/100g tissue) within sheep. For between sheep analyses, the amount of isotope retained in the skin was adjusted to account for the quantity of isotope infused and liveweight (Equation 6.2).

Equation 6.2.

$$\text{Isotope retained in the skin (CPM / mg tissue)} = \frac{\text{radioactivity of skin}}{\text{total counts infused per liveweight}^{0.75}}$$

The mean and standard error of the mean were routinely calculated with data obtained from samples within sheep and then from sheep within strains.

Analysis of variance statistics were performed with the Super ANOVA computer software package (1989-1990, Abacus Concepts, Inc, Berkeley, California). These statistics were used to test the effects of blood vessel, time of sampling and strain on nutrient concentration in the plasma and also to examine the effect of time of measurement and strain on blood flow through the skin.

The Students t-test was used to estimate differences in the measured characteristics between the strains of Merinos. Correlation coefficients were estimated with the Delta Graph computer software package (Professional version; DeltaPoint Inc., Monterey, California). This was used to derive the simple correlation coefficients between the measured characteristics.

Results were considered significantly different when $P < 0.05$.

6.4.2. Results

At the end of the stabilisation period, the mean liveweight of the finewool Merinos (56 ± 1.45 kg) was significantly greater than the liveweight of the strongwool Merinos (48 ± 2.1kg; P = 0.017) at the same time. All sheep consumed the total ration offered throughout the stabilisation period and had returned to normal consumption levels by four days after surgery. The collection and analysis of arterial blood prior to the start of the infusion was not possible in two finewool and one strongwool Merino as the deep circumflex arterial catheters were not patent prior to the start of the infusion in these animals. Also, the jugular catheter was displaced from one strongwool Merino during the infusion and jugular blood samples could not be obtained, thus the infusion data and nutrient analyses from that sheep have been omitted. The following results are presented with the number of sheep represented by each mean indicated in parentheses.

There was a highly significant difference in wool growth rate between the two strains of Merino sheep maintained under similar environmental conditions. During the final 14 days of the stabilisation period, the strongwool Merinos grew 63% more wool per unit area of skin than the finewool Merinos (Table 6.5). The strongwool Merinos also had a significantly greater volume of germinative tissue in the bulb ($P < 0.001$) and more germinative tissue in the skin than the finewool Merinos ($P = 0.002$; Table 6.5). The germinative volume of both the bulb and the skin was correlated to wool growth (Table 6.6). Conversely, follicle density did not differ significantly between the two strains of Merino (Table 6.5), nor was it related to wool growth (Table 6.6).

Analysis of variance indicated that blood flow through the skin at two sites did not differ between measurements made prior to the start of the infusion and four hours after infusion ($P = 0.958$), nor was there any interaction between time and strain. There was a significant effect of strain of Merino on blood flow measurements ($P = 0.004$) and when the measurements from the 0 and 4h period where combined for each strain, the strongwool Merino had significantly greater flow through the skin than the finewool Merino (Table 6.5). The mean blood flow through the skin for each sheep was significantly correlated with both wool growth and the volume of germinative tissue in both the bulb and the skin (Table 6.6).

Table 6.5. Wool production per unit area of skin (wool growth), follicle density, volume of germinative tissue in the bulb (bulb volume), total germinative volume of the skin (total germ. volume) and blood flow through the skin of finewool and strongwool Merinos (Means ± s.e.m. in parentheses)

	Finewool (n = 4)	Strongwool (n = 3)	*P* =
Wool growth (g x 10^{-3}/cm^2/d)*	0.46 (0.009)	0.75 (0.054)	0.002
Follicle density (mm^{-2})	41.0 (4.38)	50.6 (4.98)	0.199
Bulb volume (μm^3 x 10^4)	15.1 (0.56)	24.3 (0.88)	< 0.001
Total germ. volume (μm^3 x $10^5/mm^2$)	62 (5.9)	124 (10.6)	0.002
Blood flow (ml/min/100g tissue)#	2.3 (0.19)	4.5 (0.81)	0.02

* Wool production per unit area of skin was measured over the last 14 days of the stabilisation period

Mean of four measurements per sheep, two of which were made prior to the start of the infusion and two measurements made after 4 hours of infusion

Table 6.6. Relationships between wool production per unit area of skin (wool), follicle density (FD), volume of germinative tissue in the follicle bulb (bulb vol) and total germinative volume of the skin (germ vol) and blood flow through the skin (flow) (Simple correlation coefficients)

	FD	Bulb vol	Germ vol	Flow[1]
Wool	0.28	0.96**	0.73*	0.89**
FD			-	0.42
Bulb vol			-	0.85**
Germ vol				0.73*

1 Mean of four measurements per sheep, two of which were made prior to the start of the infusion and two measurements made after 4 hours of the infusion

* $P < 0.05$; ** $P < 0.01$

6.4.2.1. Glucose flow and ^3H retained in the skin

The analysis of variance indicated that the concentration of glucose in deproteinised plasma was not affected by the strain of Merino (P = 0.480), the time of sampling (P = 0.143) nor the blood vessel from which it was taken (P = 0.956). There were also no interactions between any of the factors. The mean concentration of glucose in the arterial samples were used in the present analyses.

The amount of glucose flowing through the skin per unit time was significantly greater in the strongwool Merinos than the finewool Merinos (Table 6.7) and glucose flow was highly correlated with wool growth (Fig. 6.4; $r = 0.91$, $P < 0.01$).

There was no difference in amount of ^{3}H retained by the skin between the two strains of Merinos (Table 6.7). The amount of ^{3}H flowing through the skin was not related to the amount of ^{3}H retained by the skin when data from both strains were combined ($r = 0.06$; Fig. 6.5) nor was there any relationship within the strongwool Merinos but there was a significant positive relationship in the finewool Merinos ($r = -0.35$ and $r = 0.66$ for the strongwool and finewool Merinos, respectively.). The amount of ^{3}H retained by the skin was not correlated with total germinative volume of the skin (Fig. 6.6; $r = 0.17$; $P > 0.1$).

Table 6.7. Concentration of glucose in deproteinised arterial plasma (glucose concentration), amount of glucose flowing through the skin (glucose flow) and amount of ^{3}H retained in the skin expressed as a proportion of the amount of isotope infused into the whole body (^{3}H retained in the skin) of finewool and strongwool Merinos (Means ± s.e.m. in parentheses)

	Finewool (n = 4)	Strongwool (n = 4)*	*P* =
Glucose concentration (μg/ml)	556 (7.4)	539 (10.0)	0.197
Glucose flow (mg/min/100g tissue)	1.3 (0.10)	2.6 (0.40)	0.019
^{3}H retained in skin	0.73 (0.074)	0.82 (0.090)	0.467

* n = 3 for ^{3}H retained in skin

6.4.2.2. Cystine flow and ^{35}S retained by the skin

The analysis of variance indicated that the concentration of cystine in deproteinised plasma was not affected by strain of Merino ($P = 0.399$), the vessel from which the sample was taken ($P = 0.422$), nor the time of sampling ($P = 0.067$). There were no interactions within the analysis. The concentration of cystine in plasma taken from the carotid artery was used in the current analyses (Table 6.8).

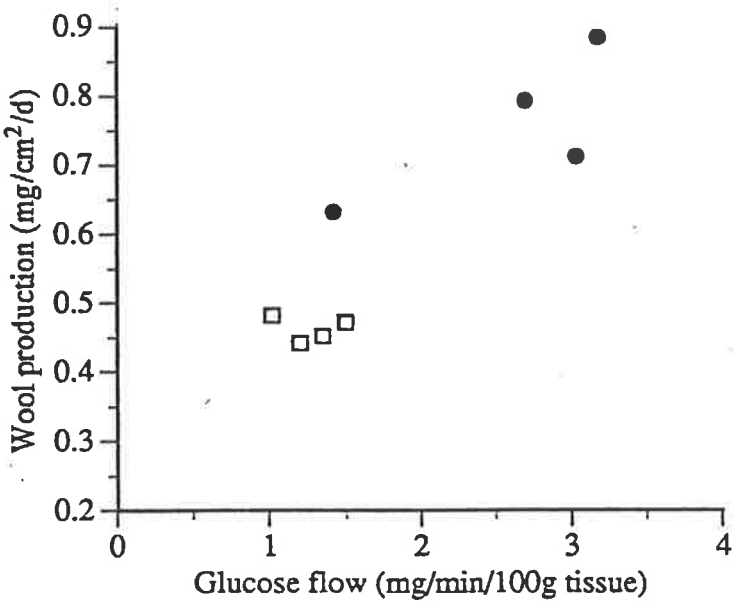


Figure 6.4. The relationship between the concentration of glucose flowing through the skin (glucose flow) and wool production per unit area of skin of the finewool (□) and strongwool (•) Merinos after infusion of ^{3}H-glucose. ($r = 0.91$: $P < 0.01$)

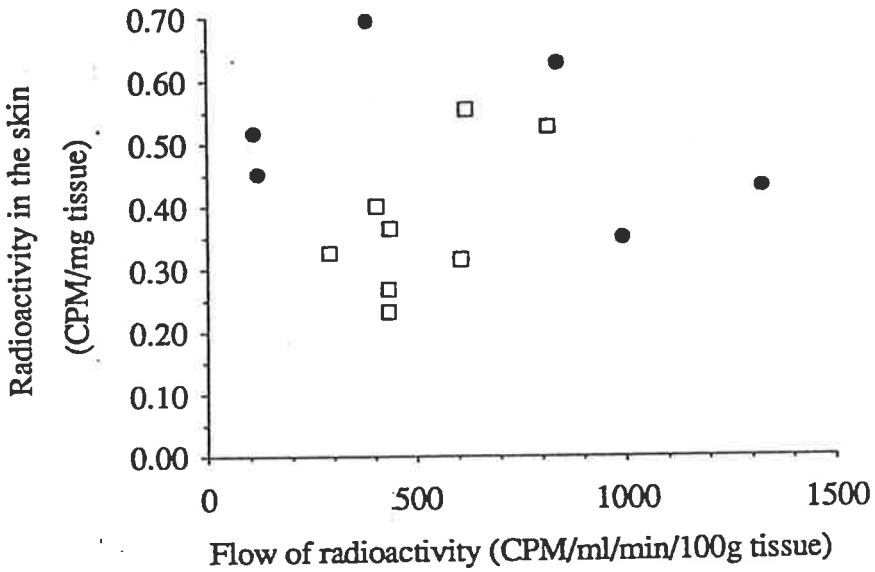


Figure 6.5. The relationship between the amount of ^{3}H-radiaoactivity flowing through the skin (flow of radioactivity) and the amount of ^{3}H-radioactivity retained by the skin of the finewool (□) and strongwool (•) Merinos after infusion of ^{3}H-glucose. (combined r = 0.06)

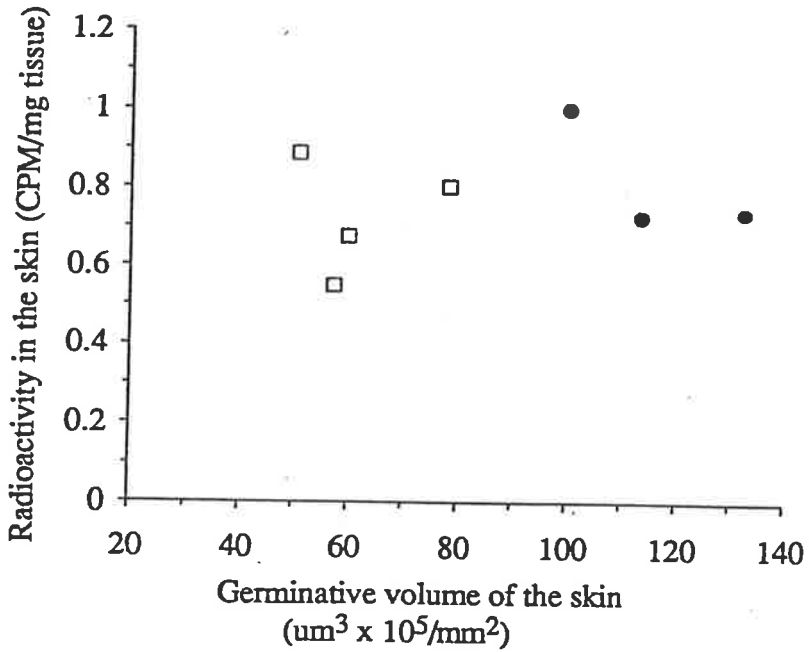


Figure 6.6. The relationship between the total amount of germinative tissue in the skin and the amount of ^{3}H-radioactivity retained by the skin of the finewool (□) and strongwool (•) Merinos after infusion of ^{3}H-glucose. (combined r = 0.17)

There was significantly more cystine flowing through the skin of the strongwool Merinos than the finewool Merinos (Table 6.8) and wool growth was related to the amount of cystine flowing through the skin when both strains were combined (Fig. 6.7, r = 0.90, P < 0.01).

Table 6.8. Concentration of cystine in deproteinised arterial plasma (cystine concentration), amount of cystine flowing through the skin (cystine flow) and the amount of ^{35}S retained by the skin expressed per amount of isotope infused (^{35}S retained in skin) of finewool and strongwool Merinos (Means ± s.e.m. in parentheses)

	Finewool (n = 4)	Strongwool (n = 4)*	*P* =
Cystine concentration (μg/ml)	1.7 (0.04)	1.8 (0.06)	0.713
Cystine flow (μg/min/100g tissue)	4.2 (0.66)	8.9 (2.50)	0.031
^{35}S retained in skin (CPM/mg tissue)	0.21 (0.002)	0.21 (0.002)	0.860

* n = 3 for cystine uptake determination

There was no difference between strains in the amount of ^{35}S retained in the skin when expressed relative to the amount of isotope infused (Table 6.8), nor was there any relationship between the amount of ^{35}S-cystine retained in the skin and the amount of ^{35}S-cystine flowing through the skin within strains (r = 0.61 and r = -0.48 in the finewool and strongwool Merinos, respectively) or when the data from the two strains were combined (Fig. 6.8; r = 0.07; P > 0.1). The amount of ^{35}S-cystine retained by the skin was not correlated with total germinative volume of the skin (r = -0.05; P >0.1).

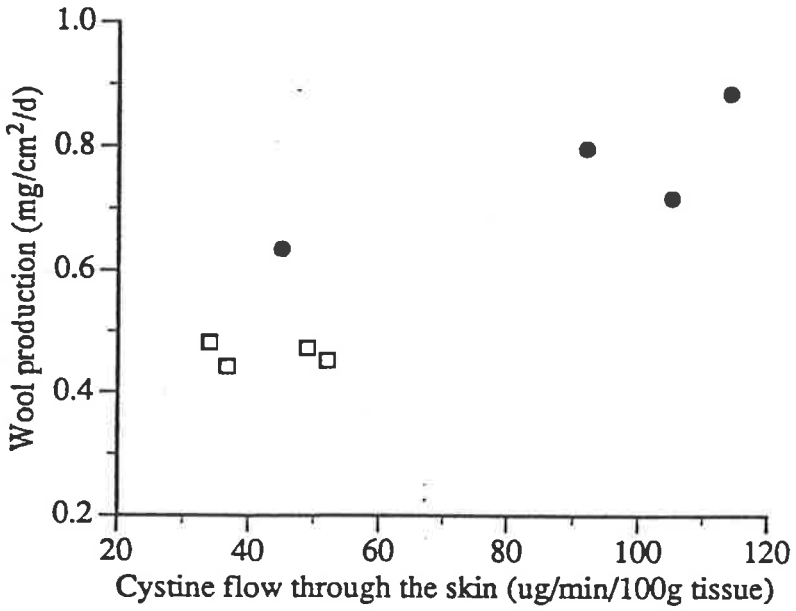


Figure 6.7. The relationship between the concentration of cystine flowing through the skin (cystine flow) and wool production per unit area of skin of the finewool (□) and strongwool (•) Merinos after infusion of ^{35}S-cystine. (r = 0.90: P < 0.01)

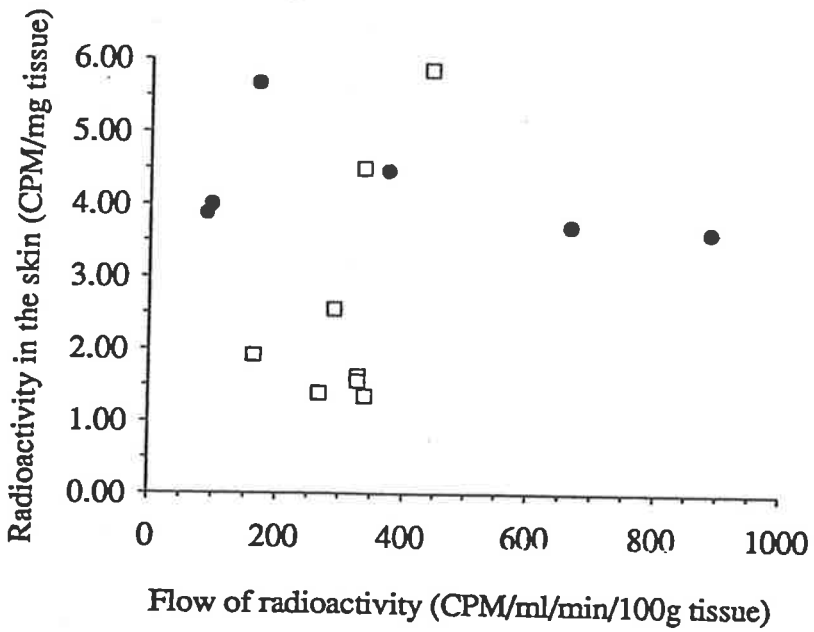


Figure 6.8. The relationship between the amount of ^{35}S-radiaoactivity flowing through the skin (flow of radioactivity) and the amount of ^{35}S-radioactivity retained by the skin of the finewool (□) and strongwool (•) Merinos after infusion of ^{35}S-cystine. (combined r = 0.07)

6.4.3. Discussion

The present work was a preliminary investigation into the *in vivo* uptake of nutrients from the plasma by the skin and follicles. The results indicated that there was no difference in the amount of ^{35}S-cystine and ^{3}H-glucose retained by the skin and follicles between strains of Merinos and, surprisingly, the retention of isotope was not related to amount of isotope flowing through the skin. Given that there were a number of equivocal assumptions made during the calculation of the isotope data (Section 6.4.1.6), the results must be examined with caution. Nevertheless, the experiment was undertaken to examine whether blood flow is influencing wool growth simply by supplying the skin with more nutrients. No attempt was made to account for the flow of isotope into and out of the skin during the infusion, the movement of which is dependant on pool sizes and rate constants. It is acknowledged that these are likely to confound the present results and may also contribute to differences in nutrient uptake and utilisation between sheep which were not detected in the current trial.

The results from the present experiment confirm the observations of Chapters 2 and 3, that blood flow and the volume of germinative tissue in the bulb and skin are significantly related to wool growth (Table 6.6). It was shown for the first time that blood flow through the skin is related to the total volume of germinative tissue in the skin. This suggests that blood flow and wool growth are related directly and also indirectly via the volume of germinative tissue in the skin.

Both the amount of ^{3}H and ^{35}S-cystine retained by the skin were independent of the amount of isotope passing through the skin. These results do not tend to support the hypothesis that nutrient uptake is related to the amount of nutrient supplied to the skin (i.e. the product of blood flow and nutrient concentration in the blood). This was unexpected, since it has often been proposed that by increasing the blood flow, and subsequently increasing the amount of nutrients available to the skin, the amount of nutrient incorporated by the skin will increase (Black & Reis, 1979; Williams 1987; Lee & Harris, 1991). Thus, the function of the high rates of blood flowing through skin is still unresolved since it appears that the additional amount of radiolabelled compounds passing through the skin, as a consequence of the higher blood flow rate, is not being retained by the skin. A limitation

of the current experiment was that only total radioactivity was measured which may mask differences in pool sizes and rate constants of nutrient flow between sheep. The ratio of free cystine to cystine bound to plasma proteins does not differ between the Fleece-plus and Fleece-minus flocks (Williams *et al.*, 1972a) suggesting that it is unlikely that differences in pool sizes within the skin between sheep will influence nutrient uptake results. If actual nutrient uptake had been estimated (by estimating the specific radioactivities of glucose and cystine in the plasma and skin) differences in nutrient uptake between strains may have been identified. Thus, a more detailed examination of the nutrient uptake mechanisms and kinetics of the skin of different strains of Merinos needs to be conducted.

Retention of radiolabelled cystine and glucose retention by the skin appears not to be limited by the supply of nutrients to the skin, rather the amount of nutrient supplied appears to be in excess of the requirements for the skin and follicle under 'normal' conditions. It is proposed that blood flow may have a role in determining wool production when nutritional conditions are severe. That is, in periods of low nutrient availability to the sheep, more nutrient is transported to the skin of high-wool producers than low-wool producers due to the greater blood flow rates of high-wool producers. This, then, maintains the large volume of germinative tissue in the skin and high levels of wool growth.

Although there was no relation between ^{3}H in the skin and ^{3}H supply when the data from the two strains was combined, there was a significant correlation between ^{3}H supply and ^{3}H in the skin of the finewool Merinos when infused with ^{3}H-glucose. This suggests that blood flow may have been limiting the supply of ^{3}H-glucose to the follicle in these sheep. It is unlikely that by increasing the blood supply to the skin of the low-wool producing sheep wool growth will also increase, since there was a poor relationship between wool growth and ^{3}H uptake in the present experiment. Alternatively, the difference in ^{3}H uptake between the two strains could be due to a difference in the functioning of the follicle. For example, nutrient uptake by the follicles may be via an active-transport process in the strongwool Merinos, in contrast to a simple diffusion mechanism in the skin of the finewool Merinos. This scenario seems unlikely since it would be unusual for such major differences in follicle function to exist within a breed of sheep.

^{35}S-cystine uptake was not related to the volume of germinative tissue in the skin. This result was not unexpected. Cystine is primarily incorporated into the keratinisation zone of the follicle (Chapter 5) and the quantity of cystine utilised by the follicle would be determined by the type and amount of protein produced during fibre keratinisation. This process would not necessarily be related to the volume of germinative tissue in the bulb. To further understand the relationship between cystine uptake and its use, it would be necessary to investigate the rate and processes of keratinisation in the two strains of Merinos.

The concentration of glucose in the arterial and venous plasma samples did not differ, so in future work it will be possible to use the easier jugular sampling technique for the analysis of plasma glucose. The arterial concentration was used since it was readily available in the present study and gave a direct representation of the nutrient concentration flowing through the skin. The glucose concentration were in the same range as those obtained in Romney wethers (409 - 630μg/ml; Harris *et al.*, 1989), although these authors noted a greater range of concentrations between sheep than in the current experiment. The plasma concentrations were slightly less than those recorded for Corriedale ram lambs (707 - 720μg/ml; Cottle, 1988) and Fleece-plus/Fleece-minus ewes (686-727 μg/ml; Hough *et al.*, 1988). These differences in glucose concentration may simply represent differences in environmental and experimental conditions, thus it appears that plasma-glucose concentrations do not differ between sheep of different wool growing capacities.

The plasma concentration of cystine was similar to that reported by others (Williams *et al.*, 1972a,b; 1986; Hough *et al.*, 1988; Lee & Harris, 1991). The cystine concentration did not differ between the plasma of finewool and strongwool Merinos, which is in agreement with studies in Romneys (Lee & Harris, 1991) but contrary to previous studies of plasma cystine concentrations in Merinos (Williams *et al.*, 1972a,b; 1986; Williams, 1976, 1984; Hough *et al.*, 1988). The disagreement between the results from the Fleece-plus/Fleece-minus flocks and the other groups of sheep is open to conjecture, but may be related to the selection for differences in wool growth within a strain of Merino, which is not expressed between strains or breeds of sheep.

One of the deficiencies in this experiment was the assumption that all of the ^{3}H and ^{35}S detected by the ß-counter in the deproteinised plasma was associated with either glucose and cystine and thus available for uptake by the skin (Section 6.4.1.6). It was further assumed that the sheep would all have similar whole body and skin pool sizes and that if all of the radioactivity is not available for uptake, then the proportion available would be similar between sheep. It is recognised that these are very general assumptions but it was decided that this method of estimating total radioactivity was the simplest way to determine the radioactivity associated with cystine and glucose, and thus was adequate for this **preliminary** investigation into nutrient uptake. No reports pertaining to the distribution of glucose-related products and specific radioactivities were found. It is known that when ^{35}S-cystine is infused into sheep, the ^{35}S in the plasma is predominantly associated with plasma proteins whereas ^{35}S associated with free-cystine is the second major component of the plasma (Williams *et al.*, 1979). If the plasma proteins are removed, i.e. by precipitation with sulphosalysilic acid, then it can be assumed that free cystine is the major ^{35}S component. For the present experiment, it was therefore reasonable to assume;

a) that the ^{35}S activity detected in deproteinised plasma was mainly associated with cystine available for uptake by the skin, and

b) that the proportion available was not dissimilar between sheep.

General Discussion

What is past is prologue

Oliver Stone, 1991

Director of the movie JFK

CHAPTER 7. GENERAL DISCUSSION

In this thesis an attempt was made to elucidate the possible mechanisms responsible for, and associated with, the wool production differences which exist between sheep maintained in similar environmental conditions. Two strains of Merinos were chosen. These provided a compromise between yielding sufficient variation in wool growth rates to identify the determinants whilst ensuring similar wool 'types'. The latter point is important, as extrapolation of results from widely-divergent genotypes (e.g. Lincoln and finewool Merino) is unlikely to be meaningful due to differences in the basic characteristics of the wool and its mechanism of production (e.g. medullated versus unmedullated fibres, seasonal growth versus non-seasonal growth). It is not strictly valid to designate the differences between strains as genetic differences since maternal and pre-experimental conditions were not controlled. It is likely, however, that the differences were largely genetic in origin because;

(i) the comparisons were made in similar environments,

(ii) the 'stabilisation' period prior to each experiment was long enough (≥46 days) to remove transient environmental effects (Hynd, 1982),

(iii) maternal and environmental effects on wool growth are generally small (~5-10%; reviewed by Corbett, 1979) in relation to the two-fold differences between groups of sheep reported in this thesis; and

(iv) the strongwool and finewool Merinos have undergone differential selection for almost 200 years (Cottle, 1991).

The major determinant of wool growth was clearly the amount of tissue in the skin capable of producing wool (i.e. germinative tissue). This occurred in all flocks of sheep examined, including the 'primitive' Camden Park Merinos, the randomly-selected strongwool Merinos and the modern, commercial finewool and strongwool Merinos. In all cases, the total volume of germinative tissue in the skin was highly correlated with wool growth. The relative contribution of the two components of germinative tissue volume, namely follicle density and the average volume of individual bulbs, differed depending on

the flock examined. Follicle density was negatively correlated with bulb volume in some groups of sheep (Experiment 1; Fig. 2.2.), whereas bulb volume was not always significantly different between groups of sheep which produced different quantities of wool (Experiment 2; Table 2.11.). This anomaly suggests that wool production from a given area of skin is determined by the follicle **population** rather than differences in the functioning of **individual** follicles *per se*.

Morphological attributes of follicles such as curvature, depth, number and the ratio of secondary follicles to primary follicles are highly correlated with wool production levels (e.g. Brown & Turner, 1968; Nay & Hayman, 1969; Nay, 1970; Barlow, 1974; Jackson, *et al.*, 1975; Gregory, 1982). It has been suggested that these are controlled by genes or group of genes which ultimately determine wool production (Jackson, *et al.*, 1975). Jackson *et al.* (1975) concluded that there are three independent pathways of genetic control (Fig. 7.1.). The first (F_I) is via the S/P ratio and controls fibre density, the second (F_{II}) is via straight deep follicles which control the length of the fibre and thirdly (F_{III}), is via either primary or follicle group density which controls fibre diameter and the density of the primary follicles. Jackson and his co-workers suggested that these are three separately acting, but possibly linked, sets of genes. The finding in the present work, that the volume of germinative tissue is highly correlated with wool growth, can also be incorporated into the model (Fig. 7.1.). The F_I pathway would obviously encompass the density component of the total volume of germinative tissue in the skin, whereas F_{III} may be the pathway controlling the size of the bulbs. Fibre diameter is associated with bulb size (Schinckel, 1961; Henderson, 1965) and diameter also tends to be negatively correlated with follicle density (Brown & Turner, 1968; Turner *et al.*, 1970; Barlow, 1974). This suggests that the **total** volume of germinative tissue in the skin would be determined by the degree to which each pathway is operating. The way the two groups of genes (G_I and G_{III}) interact may control the role of density and bulb volume in determining the total volume of germinative tissue and thus, the amount of wool produced. For example in the ANAMA flock (Experiment 2.), the selection criteria of increased fleeceweight with a ceiling on fibre diameter has placed emphasis on the F_I pathway. This has resulted in an increased follicle

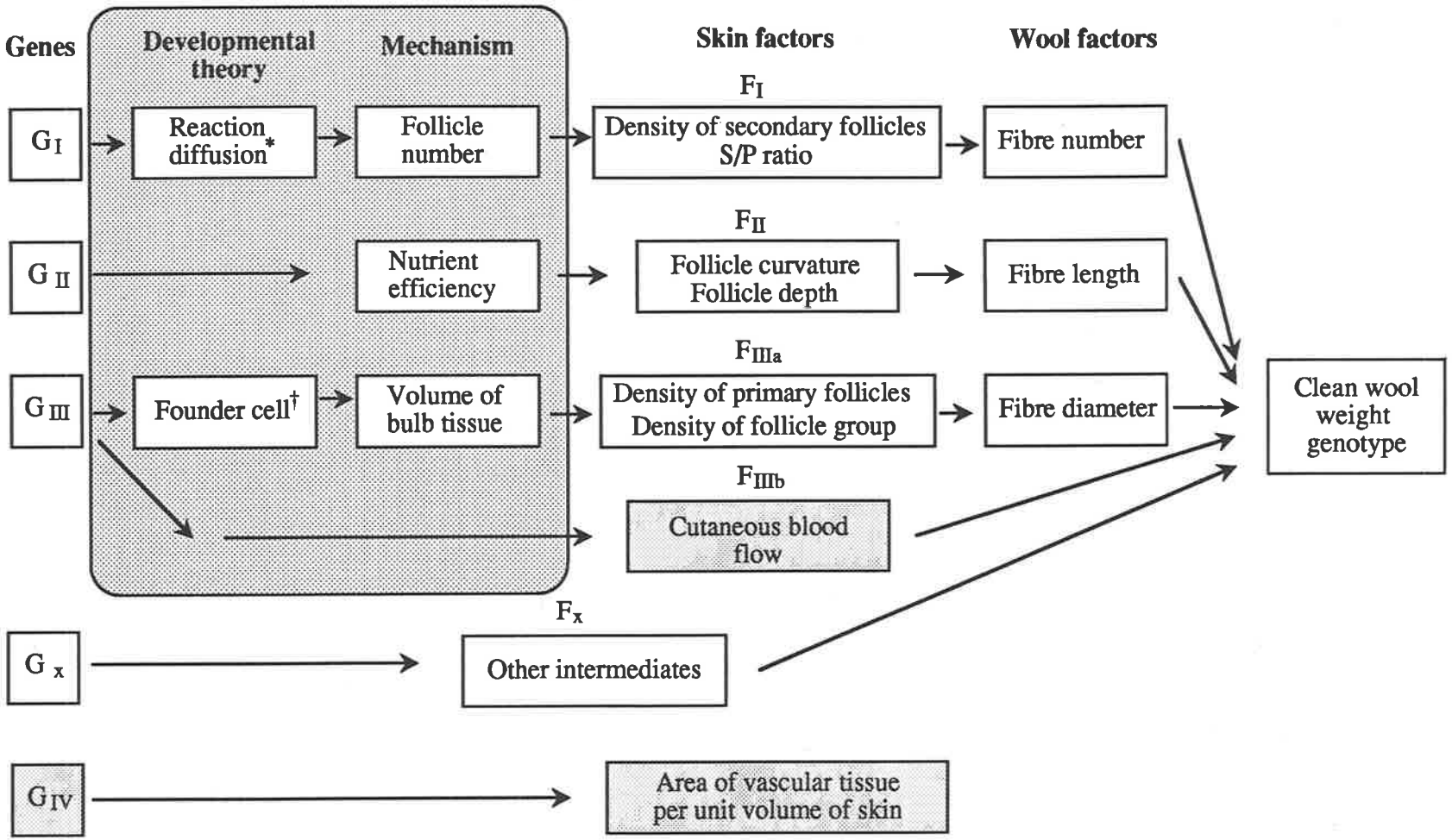


Figure 7.2. Modified model for control of clean wool weight genotype via independant follicle factors with reference to the possible therories and mechanisms by which the genes act in relation to components of clean wool weight (Adapted from Jackson *et al.* (1975)). Unshaded area is that proposed by Jackson *et al.* (1975) ☐

* proposed by Nagorcka and Mooney, 1989

† proposed by Moore *et al.*, 1989

density but very little change in bulb volume, and thus fibre diameter, in the stud flock compared to the ramdomly-selected flock.

The G_I and G_{III} groups of genes would account for almost all the variation in wool growth between sheep. In fact, in Experiment 1., 82% of the variance was accounted for by bulb volume and follicle density (Section 2.3.6.). When other follicle attributes such as cell division rate, the proportion of bulb cells entering the fibre and the size of the cortical cells were included in the regression, they did not make a significant contribution to the variation in wool production between sheep. This suggests that it is the total germinative volume of tissue which is important in wool production and any differences in the other factors, such as cell division rate and cortical cell volume, only make a minor contribution to differences in wool production between sheep. To attempt to control wool production levels or alter the genetic capacity of sheep it is important to develop an understanding of the mechanisms which control the amount of germinative tissue in the skin.

The distribution of the follicles is determined prior to birth (Carter & Hardy, 1947; Hardy & Lyne, 1956a) with very little change after birth, thus the development of follicles is important in determining the the potential level of wool production. In mammalian follicles a number of epithelial-mesenchymal interactions occur during follicle development. The control signals have been investigated by dermal-epidermal recombination experiments (reviewed by Sengel, 1986; and Hardy, 1992). The first stage of follicle development is initiated by the dermal cells which send a signal to the epidermal cells which, in turn, respond by forming an appendage (Kollar, 1970; Dhouailly, 1973). This is followed by an epidermal message that appears to cause the rearrangement of the dermal cells into a dermal papilla (Oliver, 1967). A second dermal message is then transmitted from the dermal papilla to the adjacent epithelial cells which are stimulated to divide rapidly and form a hair follicle (Cohen, 1961; Oliver, 1970).

The mechanism whereby the number and size of the follicles is determined is, as yet, unknown. Several theories have been suggested. Moore and Jackson (1984) proposed that the foetus contains a specific number of dermal 'founder' cells committed to follicle formation prior to initiation. The distribution of these cells is proposed to be under some form of genetic control. The final fibre size is also proposed to be a function of the number

of cells that engage in initiation of each follicle. The number of follicles that are formed and the sizes of their prospective fibres is a consequence of the manner in which the 'founder' cell population is utilised (Moore *et al.*, 1979). They suggested that this process was under a single developmental mechanism and that the total number of 'founder' cells is genetically-controlled. It is proposed that the founder cell theory is involved in the F_{III} pathway (Fig. 7.1.). The G_{III} group of genes may be involved in the epidermal message which causes the arrangement and number of dermal cells to form a dermal papilla. This, in turn. determines the volume of germinative tissue in the bulb and resulting fibre diameter (Fig. 7.1.).

The reaction-diffusion theory provides a descriptive explanation for the events in follicle initiation and distribution (Nagorcka & Mooney, 1989; Section 1.3.1.). To incorporate this theory into the general scheme for the determination of the level of wool production, it is proposed that the G_I group of genes stimulate the production of the morphogens which, in turn, determine the distribution and density of the follicles within the skin (Fig. 7.1.). Nagorcka and Mooney (1989) described a mechanism in which an unknown dermal factor (S) switches on the reaction-diffusion morphogens (X and Y). These are confined to the epidermis. A localised high concentration of X will theoretically cause epidermal appendages to form, in agreement with the theory described earlier pertaining to the first signal for follicle initiation being of dermal origin. The G_I group of genes may control the concentration and location of S, which in turn controls the distribution of the morphogens and the subsequent location and density of the follicles.

The F_{II} pathway relates the efficiency of follicle functioning to follicle morphology since straight, deep follicles are more efficient than shallow, curved follicles (Jackson, *et al.*, 1975). The follicles of high-wool producing sheep tend to be deeper and longer than those of low-wool producers (Nay, 1966), which may lead to a closer association between the blood vessels and the follicles of the strongwool Merinos than in the finewool Merinos. The access of the follicles to nutrient is enhanced, hence the strongwool Merinos may have a greater rate of nutrient uptake than the finewool Merinos simply due to the morphology of the skin, as proposed by Nay (1966). However, it was found that there was no difference in the ability of the follicles to incorporate ^{35}S-cystine from an extracellular pool between the

finewool and strongwool Merinos (Sections 5.3.4 and 6.4.2.2). The amount of ^{35}S-cystine retained by the skin was not associated with the amount of nutrient available to the skin, nor to blood flow, suggesting a difference between follicles of different sheep in their ability to utilise similar amounts of cystine to produce different quantities of wool. The G_{II} group of genes may control the type of keratins produced and possibly the structure of the follicle. For example, it is known that cystine activates a ultra-high sulphur keratin gene resulting in an increase in the production of paracortex (Fratini *et al.*, in press). If this gene (as part of the G_{II} group of genes) is not present or non-functional in high-wool producing sheep, then the cystine would be used for the production of orthocortex, and thus more wool could be produced because of the lower sulphur content of the orthocortex (Rogers, 1959; Kaplin & Whitely, 1978; Orwin *et al.*, 1984; Powell & Rogers, 1986). The apparent activation of the ultra-high sulphur genes appears to be highly variable between sheep when subjected to different levels of cystine (Hynd, 1989b), further implying a potential genetic role in efficiency of nutrient use. Williams (1987) also posed the question of whether the genes controlling the synthesis of the ultra-high sulphur proteins are present in high-wool producing sheep.

^{3}H-Glucose uptake was not related to wool growth and similar amounts of ^{3}H were retained by the skin of the two strains of Merino despite differences in wool growth. This anomaly could relate to a difference in glucose utilisation between different types of cells, e.g. low wool producers tend to have a high proportion of inner root sheath cells and/or paracortical cells (Butler & Wilkinson, 1979; Orwin, 1989; Hynd, 1989a), both of which may require more energy for metabolism, differentiation and protein synthesis. Alternatively, the G_{II} group of genes may determine the degree to which energy is produced by different metabolic pathways. The utilisation of glucose anaerobically is apparently inefficient due to the low yield of ATP (2 moles of ATP per mole of glucose) compared with that obtained be aerobic oxidation via the tricarboxylic acid cycle (36 moles of ATP per mole of glucose). If high-wool producing sheep were capable of utilising some glucose aerobically due to metabolic differences and/or increased oxygen supply through either increased blood flow or closer proximity to the vessels, less glucose would be required for wool growth. Lactate is the breakdown product of anaerobic glycolysis and

was higher in the plasma of Fleece-minus sheep than Fleece-plus sheep (Hough *et al.*, 1988), suggesting that there may be a difference in energy metabolism between genetically-different sheep. A large amount of variation was found in the utilisation of glucose and oxygen and the output of lactate by the isolated cutaneous patch of four Romneys (Harris *et al.*, 1989). Wool growth was not measured, thus conclusions pertaining to the relationships between energy metabolism and wool growth cannot be made. Nevertheless, there was sufficient variation between the sheep to suggest that there may be differences in the mechanisms of energy metabolism.

The relationship between blood flow and nutrient uptake by the skin and follicles of sheep differing in wool growth remains equivocal. The results of Experiments 3 and 6 indicate a clear relationship between skin blood flow and wool production, as predicted by the model of Black and Reis (1979). In fact, there was a significant relationship between the amount of nutrient flowing to the skin and wool production, but the uptake of radiolabelled nutrients by the skin appeared to be independent of nutrient supply (Sections 6.4.2.1 and 6.4.2.2). This suggests that blood flow is not controlling wool growth via nutrient supply. There may be an alternative role for blood flow in determining wool growth, for example in the supply of oxygen to the follicles or differences in the temperature of the follicle microenvironment.

The oxygen requirement for wool growth has not been examined and the role of oxygen in hair growth is uncertain. Bullough and Laurence (1958) found that there was no mitotic activity in the skin of mice when the skin was incubated *in vitro* in the absence of oxygen. In contrast, it was proposed that hair growth is largely independent of short-term changes in oxygen supply (Montagna & Parakkal, 1974). In support of this is the observation that energy is predominantly metabolised anaerobically to lactate by the glycolytic Embden-Meyerhof pathway in hair follicles (Adachi & Uno, 1968, 1969; Philpott & Kealey, 1991). In the wool follicle, both the aerobic and anaerobic pathways of glycolysis are operational (Leng & Stephenson, 1965). These results suggests that follicles are capable of producing ATP both aerobically and anaerobically. Wool production may be affected by blood flow which influences oxygen levels of the skin and its follicles and thus the efficiency of energy production.

The blood vessels of the skin have a role in whole body thermoregulation. This may affect the temperature of follicle and thus change the kinetics of the biochemical reactions in the follicles. It is known that high skin temperatures stimulate wool growth, whereas low skin temperature decreases wool growth, but it has been assumed that these temperature changes are due to differences in blood flow to the skin (Cockrem, 1962; Doney & Griffiths 1969; Jolly & Lyne, 1970; Lyne *et al.*, 1970). Low temperatures (4°C) do inhibit the metabolism of the skin and wool follicles in vitro (Experiment 5) and it is known that low temperatures also affect the uptake of amino acids in bacteria (e.g. Piperno & Oxender, 1968; Kay & Gronlud, 1969). Whether a slight change in the temperature of the microenvironment resulting from differences in blood flow and vessel distribution is sufficient to affect wool growth is unknown, but may play a small role in determining the rate of wool production.

It is concluded that blood flow is simply an accompanying factor associated with wool growth, rather than directly determining wool growth, in most situations. Blood flow is highly correlated with the volume of tissue both in the bulb ($r = 0.85$) and in the skin ($r = 0.73$; Section 6.4.2.). It is proposed that blood flow is controlled by the same group of genes (G_{III}) as the those which control the volume of bulb tissue (Fig. 7.1.). When the volume of germinative tissue is determined *in utero*, a corresponding flow rate is also determined for maintenance of this tissue. A possible mechanism may be via growth factors which are capable of stimulating both angiogenesis and folliculogenesis. The flow rate may be controlled by the size and number of individual vessels or it may be controlled by the degree of vasodilation or vasoconstriction within the skin. The mechanism for the determination of the amount of blood flowing through the skin is unknown and further understanding of this may indicate the role of blood flow in determining the level of wool production.

There was no relationship between the total area of vascular tissue per unit volume of skin with blood flow, follicle density or wool production (Chapter 4). It is proposed that a fourth group of genes (G_{IV}) control the volume of vascular tissue per unit volume of skin but do not affect the clean wool weight genotype (Fig. 7.1). The G_{IV} group of genes are likely only to be operational during foetal development and wound healing, i.e. at similar

times to when G_I and G_{III} are active. Angiogenesis occurs during foetal development whereas capillary proliferation is rare in adult organisms under physiological conditions. The G_{IV} group of genes may interact with the G_{II} group of genes. For example, as the follicles are becoming longer and deeper within the skin, the follicles incorporate vascular tissue into the papilla. This was observed in the strongwool Merinos in Experiment 4 and is common in other species, particularly those with large dermal papillae (rat - Durward & Rudall, 1949, 1958; calf - Goodall and Yang, 1954; human - Montagna & Ellis, 1958).

Selection for superior wool producing genotypes

Traditional genetic approaches to improving wool production have resulted in slow gains in production, partly due to the antagonistic correlations between traits such as fibre diameter and follicle density (Section 1.4.2.). Skin-based selection procedures have been suggested as a means of increasing the rate of genetic gain by providing a selection process at an earlier age and thereby decreasing the generation interval. Furthermore, skin-based selection procedures have the potential to control wool growth at the level of production, i.e. the follicle. Thus, it is appropriate that the results of this work are discussed in terms of the usefulness of the findings as selection criteria for wool production.

The laser Doppler velocimeter to estimate blood flow through the skin of sheep, may be useful for the identification of high wool-producing sheep. It is concluded that blood flow through the skin is not a major determinant of wool growth but may be useful as an indirect selection trait. Furthermore, selection for increased blood flow in sheep in conjunction with characteristics such as increased germinative tissue in the skin may provide a further advantage by overcoming competition between follicles (Fraser, 1953, 1954). A combination of tandem selection for blood supply and high germinative volume would allow increased production of low diameter (high density) wool because the larger number of follicles in the skin would each continue to be served by the equivalent amount of blood as lower follicle density skin. This may also improve the quality of the wool by stabilising strength and or diameter.

The use of a combination of traits, such as total germinative volume of skin, which encompasses both follicle density and the volume of the follicle bulb (which is highly related to fibre diameter), as selection criteria may allow a rapid increase in wool growth.

This would overcome some of the problems encountered in the single selection experiments (Section 1.4.2.). The negative relationship between follicle density and the volume of germinative tissue in the bulb reported in Chapter 2 both supports Fraser's (1953) follicle competition theory and provides further evidence to Hynd's (1991) explanation of why selection for single components of wool production have been ineffective (Davis & McGuirk, 1987). Hynd (1991) suggested that there is no change in wool output since selection for one component results in an antagonistic change in another and merely moves the result up or down the regression line. He further proposed that the only effective selection program is one which increases the total volume of germinative tissue per unit area of skin - this is supported by the present results. Indeed, concurrent selection for increased clean fleece weight and decreased fibre diameter would be expected to increase the germinative volume of the skin, as indicated by the data from the ANAMA flocks in Experiment 2.

APPENDICES

APPENDIX 1. GENERAL MATERIALS AND METHODS

A1.1. Tissue processing.

Processing of the tissue was carried out in a Citadel tissue processor (Shandon Southern Products, Ltd. England) as follows;

70% (v/v) ethanol	60 min
80% (v/v) ethanol	60 min
95% (v/v) ethanol	30 min
95% (v/v) ethanol	30 min
Absolute ethanol	120 min
Absolute ethanol	120 min
1:1 Absolute ethanol : Safsolvent	60 min
Safsolvent (Ajax Chem., Auburn, NSW)	120 min
Safsolvent	120 min
Histological wax (Phoenix Scientific Ind., Norwood, SA)	120 min
Histological wax	120 min

The samples were embedded in paraffin wax using a Tissue-Tek II embedding centre (Lab-Tek Division, Miles Lab. Inc., Naperville, Il) and then sectioned on a rotary microtome (Leitz 1512, Ernst Leitz, Wetzlar, GmbH). The sections were floated into a water bath (40°C), then attatched to microscope slides with Poly-L-Lysine (Sigma Diagnostics, St. Louis, Mo.) and oven-dried (60°C) overnight.

A1.2. Staining.

A1.2.1. Deparaffinise and hydration

1. Safsolvent	20 min
2. Absolute ethanol	2 min
3. 80% (v/v) ethanol	2 min
4. 30% (v/v) ethanol	2 min

A1.2.2. Haematoxylin and eosin (and picric acid)

This method stains nuclei blue-black and the remaining tissue various shades of pink. When picric acid is included, the fibres stain yellow.

1. Deparaffinise and hydrate (A1.2.1.)	
2. Lillee Mayer Haematoxylin [1]	1.5 min
3. Running water	rinse
4. Acid alcohol (1% conc. HCl in 70% (v/v) ethanol)	dip until pink
5. Running water	10 min
6. Eosin[2]	7 sec
7. 80% (v/v) ethanol	rinse
(7a. Picric ethanol (saturated)	30 secs)
8. Absolute ethanol	2 min
9. Absolute ethanol	2 min
10. Safsolvent	2 min
11. Safsolvent	2 min
12. Mount with DePeX. and coverslip.	

1 Harris haematoxylin (5g), AlNH4(SO4)2.12H2O (50g), Glycerol (300ml), H2O (700ml), NaIO3 (1g), glacial acetic acid (20ml)
2 1% aqueous eosin Y (50ml), 95% ethanol (390ml), 1% aqueous phloxine (5ml), glacial acetic acid (2ml)

1.2.2. Methylene blue (Clarke & Maddocks, 1965)

This method stains paracortex blue and the orthocortex remains colourless.

1. Deparaffinise and hydrate (section 3.4.3.1.)	
2. Performic acid (fresh) [1]	30 min
3. H_2O	10 secs
4. Methylene blue [2]	7 min
5. H_2O	10 secs
6. Acid alcohol (1% conc. HCl in 70% (v/v) ethanol)	7 secs
7. Eosin [3]	1 min
8. 80% (v/v) ethanol	10 secs
9. Absolute ethanol	2 min
10. Safsolvent	5 min
11. Safsolvent	5 min
12. Mount with D.P.X. and coverslip.	

1 90% Formic acid (250ml), H2O2 (100ml), H2O (650ml)
2 1% (w/v) Lofflers methylene blue
3 1% aqueous eosin Y (50ml), 95% ethanol (390ml), 1% aqueous phloxine (5ml), glacial acetic acid (2ml)

1.2.3. Immunohistochemical staining of bulb cells labelled with BrdU (Hynd & Everett, 1991)

This method stains cells labelled with BrdU brown, and the surrounding tissue light purple.

1. 1.5M HCl	20 min.
2. Cold (4°C) saline / 1%Tween	Brief Rinse
3. BrdU Antibody [2]	30 min.
4. Phosphate buffered saline with Tween (PBT) [3]	Rinse
5. Biotinylated anti mouse (IgG) [4]	30 min.
6. PBT	Rinse
7. ABC (mix and stand 30 minutes before use)[5]	60 min.
8. Phosphate buffered saline (PBS)[1]	Rinse
9. 3,3'-diaminobenzidine [6]	5 min.
10. Water	Rinse
11. Counterstain lightly in Haematoxylin	10 sec.
12. Running water	10 min.
13. 80% ethanol	dip
14. Safsolvent	2 min.

1. Vial Phosphate-buffered saline (Sigma Diagnostics, St. Loius, Mo. USA) in 1l water; pH 7.4.
2. 120µl Murine MonoClonal Antibody to BrdU (Bioclone Aust. Pty Ltd, Marrickville, NSW)
   158.4µl normal horse serum (Vectastain ABC Kit, ImmunoDiagnostics, Camperdown, NSW)
   12ml PBT (as in 3 below).
3. 1% Tween 20 and 1% Bovine Albumin-RIA grade (Sigma Chemical Co., St. Loius, Mo. USA) in PBS (as in 1 above); pH 7.4 (sterile).
4. 52.8µl Anti-mouse IgG (Vectastain ABC Kit, ImmunoDiagnostics, Camperdown, NSW) in 12ml PBS (sterile) (as in 1 above).
5. 105.6µl Avidin DH (Vectastain ABC Kit, ImmunoDiagnostics, Camperdown, NSW)
   105.6µl Biotinylated Horseradish Peroxidase H (Vectastain ABC Kit, ImmunoDiagnostics, Camperdown, NSW)
   12ml PBS (as in 1 above).
6. 1ml 3,3'-diaminobenzidine (5mg/ml PBS; Sigma Chemical Co., St. Loius, Mo. USA)
   20µl H_2O_2 (30%)
   19ml PBS (as in 1 above).

APPENDIX 2. COMPARISON OF METHODS FOR THE LIBERATION OF CORTICAL CELLS

Introduction

The structure of fibre cortical cells have been studied extensively, however only a few studies have been undertaken to determine the magnitude of the cortical cells and their influence on wool production (Short *et al.*, 1965; Wilson & Short, 1979; Williams & Winston, 1987; Hynd, 1989). Generally, the methods used are destructive and the effects on the cells are varied, more suited to chemical and morphological analyses for which they were developed. These methods involve a chemical or proteolysis pretreatement before mechanical agitation to disperse individual cells from the fibre.

Hydrochloric acid is used for dissolving fibres to determine their amino acid composition (Ward, Binkley & Snell, 1955) and has been used at 60°C to determine cortical cell size (Short *et al.*, 1965; Wilson & Short, 1979; Williams & Winston, 1987). The authors recognised that there were some chemical modification and loss of material from the cells but suggested that these could be minimised by dispersing and measuring the fibres under optimal conditions and thus enable valid comparisons to be made. Alternatively, proteolytic enzymes are used to study cortical cell morphology. The enzymes dissolve the intercellular cement, the endocuticle, part of the cell membrane complex, nuclear remnants and intermacrofibrillar material of the cortex (Peters & Bradbury, 1972; Kulkarni & Bradbury, 1974). Lundgren (1956) compared the effect of hydrochloric acid with that of papain, a proteolytic enzyme, on the preparation of cortical cells. He found that the two methods produced cells of quite different composition suggesting that some modification of the cortical cells by the use of these two treatments.

Pretreatment of the fibre with formic acid minimises the level of cellular modification which occurs during acid and proteolytic treatments (Bradbury & Chapman, 1964). Cortical cells are dispersed from the fibres in formic acid by dissolution of lipid and protein material from the cell membranes between and underlying the cuticle cells (Bradbury & Chapman, 1963; 1964) and by swelling of the fibre (Bradbury *et al.*, 1966). The swelling of the fibre itself is unlikely to be solely responsible for the dissociation of the cortical cells since swelling

in water after thioglycollate and other liquids is even greater than that observed in formic acid and yet the cortex remains intact (Bradbury & Chapman, 1964; Ley *et al.*, 1985).

Agitation at room temperature after formic acid pretreatment (Bradbury *et al.*, 1966; Ley *et al.*, 1985), boiling and ultrasonication in formic acid (Bradbury & Peters, 1972; Hynd, 1989) have been found to further enhance fibre disruption. It has been suggested that formic acid may cause modification of the microfibrillar complex, implying that there is hydrolysis of peptide bonds (Bradbury & Peters, 1972), but it has been demonstrated that the treatment does not cause any other reactions other than formylation which may be reversible (Bradbury *et al.*, 1966; Peters & Bradbury, 1972).

Further studies have shown that chemical pretreatments including thioglycollic acid, acetic acid and iodoacetate should be avoided due to their low yield of whole cells and high levels of dissolved materials compared to formic acid treatment (Kulkarni & Bradbury, 1974). However there may be confounding effects in that the fibres were ultrasonicated in formic acid following the pretreatment chemical and any chemical reation between the acids may have affected the results.

Cells are generally liberated from wool fibres by treating with HCl at 60°C (Short *et al.*, 1965; Wilson & Short, 1979; Williams & Winston, 1987). Alternatively, formic acid at 60°C and ultrasonication has been used to investigate cortical cell dimensions (Hynd, 1989). However, there is limited data on the effect of these treatments on cell dimensions and the aim of this experiment was to determine the optimum conditions for cortical cell liberation and observation.

Materials and Methods

a) Direct determination of cortical cell volume

Wool was washed as described in Section 2.2.3. prior to further washing in hexane (2 x 5min) and hot water (3 x 5min), dehydration in alcohol and air drying. Approximately 5mg of clean wool was placed into small plastic vials with 5ml of treatment solution. The treatments were as follows;

1. Formic acid (90% w/v; Ajax Chemicals, Auburn, NSW) at room temperature

2. Formic acid (90% w/v; Ajax Chemicals, Auburn, NSW) at 60°C

3. 1% trypsin (to wool weight) in borate buffer* , pH 8.3, at 40°C

4. 2% trypsin (to wool weight) in borate buffer*, pH 8.3, at 40°C

5. 5% trypsin (to wool weight) in borate buffer*, pH 8.3, at 40°C

6. 10% trypsin (to wool weight) in borate buffer*, pH 8.3, at 40°C

7. 6M HCl at room temperature

8. 6M HCl at 60°C.

Treatments were examined after 15, 30, 45, 90 minutes, 1, 2, 4, 8, 16, 24, 48, 72, 96 hours and 7 days incubation. The wool was centrifuged (2000*g* for 10min, Sorvall Rotor), and the supernatant removed and replaced with water (10ml). The samples were stored at room temperature overnight and then ultrasonicated at 50 - 80W (Labsonic 1510, B.Braun) for 20 minutes. Aliquots were removed every 5 minutes and placed on a slide coated in Poly-L-Lysine (Sigma Diagnostics, St. Loius, Mo. USA).

Treatments 2, 4 and 6 were used to investigate the effects of picric acid or methylene blue staining on the dimensions of the cells† . For picric acid staining, the slides were placed in saturated picric acid (5min), rinsed in RO water, dehydrated through 30%, 80% and absolute ethanol and then cleared in safsolvent before mounting coverslips on the slides with D.P.X. The cells were stained with methylene blue by placing the slides in performic acid (1h), methylene blue (1h), rinsing in acid ethanol (1%), and dehydrating through 80% ethanol (dip), ethanol (2min), safsolvent (2min) and then mounting coverslips on the slides with D.P.X. This procedure was found to stain the cells adequately without the cells lifting off the slide.

The characteristics of the fibre and cells were recorded using a subjective analysis of the number of broken and distorted cells and the proportion of unbroken fibre. The volume of cortical cells was determined using the formula proposed by Short *et al.*, (1965). This assumes that the cortical cells are shaped like two cones placed base to base and volume can be calculated using Equation A1.

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* Borate buffer - 50ml 0.025M $Na_2B_4O_7.10H_2O$ (9.525g/L), 19.25ml 0.1M HCl; diluted to 100ml with H_2O (from Daswon *et al.*, 1986).

† Chemicals used for the stains are described in Appendix 1.

Equation A1

$$\text{Cortical cell volume} = 2 \times \frac{\text{Cell length}}{2} \times \frac{1}{3} \times \frac{\pi\,[\text{Cell width}]^2}{4}$$

The length and width of 250 cells for each treatment and stain were measured (at 1188 times magnification) and the dimensions were used to calculate cortical cell volume according to equation 8. The results were analysed by analysis of variance.

b) Indirect determination of cortical cell volume

It was proposed that by counting a representative number of cells in a known volume of wool, the average volume of cortical cells could be estimated. Thus, various weights of wool (approximately 25, 35, 45, 55 and 65mg) from one sheep were placed in plastic vials in a 60°C oven with either formic acid for 24h or HCl (6N) for 45 minutes. After treatment, the acid was replaced with water and then the solution was ultrasonicated in 5 minute intervals for a maximum of 20 minutes. The sample was then diluted to 25ml with water (50ml for the 55 and 65mg samples). An aliquot (0.5ml) was placed on a large slide which had 2 x 1mm coverslips adhered to the slide to form a chamber of exactly 1mm depth. The number of cells were counted in a known area (and thus, a known volume) and this was used to estimate volume of cortical cells according to Equations A2 to A4.

Equation A2

$$\text{Volume of wool dispersed} = \frac{\text{Weight of wool}}{\text{density of wool}}$$

where density of wool is $1.3052 g/cm^3$

Equation A3

$$\text{Number of cells per ml} = \frac{\text{Number of cells counted}}{\text{Known area x 1mm}}$$

Equation A4

$$\text{Cortical cell volume} = \frac{\text{Volume of wool[Eq. A2]}}{\text{Number of cells in 25ml}}$$

where number of cells in 25ml is [Eq. A3] x 25

The volumes of the cortical cells for each weight of wool and for each acid were analysed with analysis of variance.

Results and Discussion

a) Direct determination of cortical cell volume

Treatment of the fibres with trypsin (all percentages) resulted in very few cortical cells being liberated and there were many fibres which were not disrupted. However, the treatments in formic acid and HCL did liberate the majority of the cells leaving few whole fibres after 7 days. In fact, after 90 minutes in HCl at 60°C, almost all of the cells and fibres were completely dissolved. In both acid treatments at room temperature some fibres still remained intact. However, it appeared that incubation at 60°C in HCl for 45 minutes or formic acid for 24 hours released the most number of whole cells with few fibres not dispersed.

Staining with methelene blue appeared to distort the cells quite markedly, particularly after treatment in HCl. Alternatively picric acid did not visually appear to distort the cells.

There was a significant difference in cortical cell width (Table A2.1) and length (Table A2.2) between treatments and stains and there was also a significant interaction term. If HCl at room temperature was removed from the analysis of width (since it differed so much from the others) there was no difference between the stains effect on cell width but there still was a significant difference between pretreatment and a significant interaction term.

Table A2.1. Mean widths of cortical cells after different dispersion methods and different stains (Means ± s.d.)

	Picric acid	Methylene blue
Formic acid (60°C)	6.8 (2.0)[a]	7.4 (1.9)[c]
Trypsin (2%)	6.8 (1.7)[a]	6.5 (2.1)[d]
Trypsin (10%)	6.8 (1.8)[a]	7.3 (1.9)[c]
HCl (room temp.)	5.7 (1.9)[b]	9.0 (2.8)[e]
HCl (60°C)	6.8 (2.0)[a]	6.4 (1.8)[d]

Different letters indicate significant differences at the 5% level.

There was a highly significant difference in cortical cell volume between stains and pretreatments (Table A2.3) which is to be expected fom the varied responses of cell lengths and widths from which volume is derived.

Table A2.2. Mean lengths of cortical cells after different dispersion methods and different stains (Means ± s.d.)

	Picric acid	Methylene blue
Formic acid (60°C)	84.4 (16.7)^{a}	90.5 (15.1)^{d}
Trypsin (2%)	90.0 (17.0)^{bd}	85.9 (15.5)^{e}
Trypsin (10%)	89.1 (16.2)^{b}	85.5 (16.0)^{ae}
HCl (room temp.)	79.8 (16.5)^{c}	47.3 (12.0)^{f}
HCl (60°C)	79.5 (16.4)^{c}	56.7 (10.8)^{g}

Different letters indicate significant differnces at the 5% level.

Table A2.3. Mean volumes of cortical cells after different dispersion methods and different stains (Means ± s.d.)

	Picric acid	Methylene blue
Formic acid (60°C)	1099 (668.7)^{a}	1399 (762.8)^{d}
Trypsin (2%)	1163 (648.5)^{b}	1078 (753.2)^{a}
Trypsin (10%)	1173 (730.7)^{b}	1300 (795.3)^{e}
HCl (room temp.)	741 (492.2)^{c}	1044 (632.5)^{a}
HCl (60°C)	1047 (684.3)^{a}	659 (411.5)^{f}

Different letters indicate significant differnces at the 5% level.

b) Indirect determination of cortical cell volume

There was a large number of broken cells (which were not tallied in the cell count) and also a high proportion of unbroken fibres in both pretreatments. There was a significant difference in cortical cell volume within treatments between different initial wool volumes (Table A2.4.). However, if there was no difference in volume between the two methods used for cell dispersion. Between 45 and 55mg of wool gave the most consistant result considering that they were diluted in different amounts of water.

Table A2.4. Mean volumes of cortical cells estimated from a known volume of wool (Means ± s.d.)

Approximate wool weights	Formic acid (60°C)	HCl (60°C)
25 mg	1809 (455.4)^{a}	1802 (644.1)^{a}
35 mg	1131 (327.2)^{b}	1621 (452.4)^{ab}
45 mg	1153 (205.4)^{bc}	1352 (195.7)^{c}
55 mg	1173 (207.6)^{bc}	1305 (271.1)^{c}
65 mg	1089 (194.9)^{c}	1445 (274.6)^{bc}

Different letters indicate significant differnces at the 5% level.

Conclusion

Pretreatment of wool in formic at 60°C for 24 hours is the most efficient and disruptive method without being so severe as to affect cell volume. Staining does affect cell dimensions, however picric acid appears to be the least disruptive.

The present experiments suggests that cortical cell volume can be determined from two methods. However, it seems that actually measuring cell length and width and then calcualting volume on the assumption of the cell being shaped like two cones placed end to end, would be the most appropriate method The alternative, that of complete fibre disruption is not feasible as it appears to be impossible to completely disrupt the fibre without degrading and breaking the cells which makes it difficult to accurately estimate the number of cells in a sample.

Table A3.1.1a. Characteristics measured in Experiment 1 (Chapter 2) in six finewool Merinos at the end of the 91 day stabilisation period (mean of each measurement)

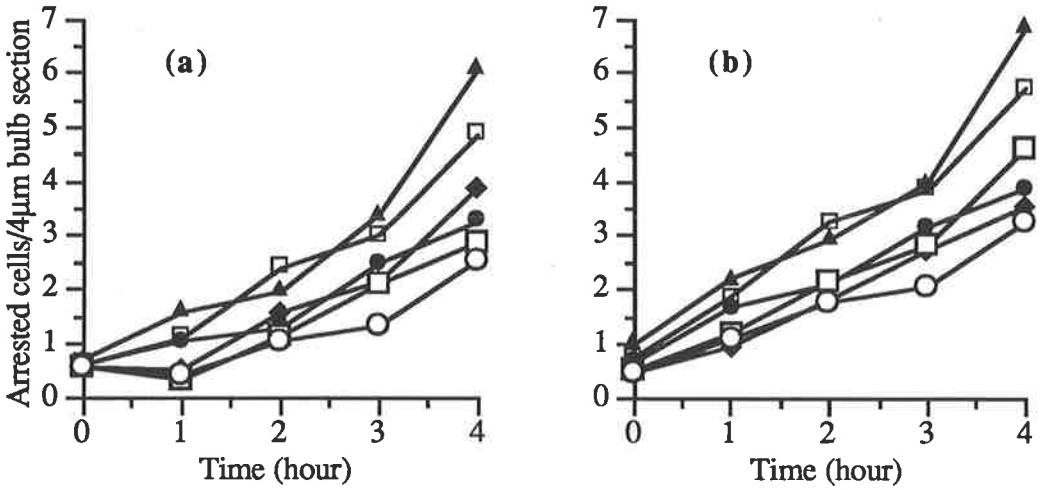
Sheep #	0	62	753	1187	1237	1242
Clean wool growth (mg / cm^2 / d)	0.452	0.329	0.424	0.525	0.746	0.667
Growth rate of fibre cortex($\mu m^3 \times 10^3$ / h)	2.885	2.045	2.391	3.468	3.905	3.564
Fibre length growth rate (µm / d)	2373.8	1800.8	2247.5	2400.8	2635.1	2355.2
Fibre width (µm)	17.2	16.7	16.2	18.7	18.9	19.1
Follicle density (mm^{-2})	47.4	44.4	55.6	41.6	67.9	56.8
Bulb volume ($\mu m^3 \times 10^5$)	0.89	0.73	0.65	1.27	0.89	0.71
Volume of individual bulb cells (μm^3)	290	166	99	145	155	201
Number of cells per bulb	308	444	654	876	574	353
Germinative volume of skin ($\mu m^3 \times 10^5/mm^2$)	42.4	32.6	36.	52.7	60.6	40.3
No. of cells produced per 4µm section per hour	0.99	1.84	2.40	1.02	1.95	2.63
No. of cells produced per bulb per hour	11.21	18.00	24.28	12.89	20.04	23.24
Turnover time of bulb (h)	27.5	24.7	26.93	68.0	28.7	15.2
Cortical cell length (µm)	96.0	84.3	96.7	97.5	96.6	88.2
Cortical cell width (µm)	6.0	5.8	5.6	5.8	5.8	5.6
Cortical cell volume (μm^3)	993.7	818.7	870.3	925.2	940.6	829.3
Number of cortical cells produced per hour.	2.90	2.50	2.75	3.75	4.15	4.30
Proportion of bulb cells entering the fibre	0.26	0.14	0.11	0.29	0.21	0.18

Table A3.1.1b. Characteristics measured in Experiment 1 (Chapter 2) in six strongwool Merinos at the end of the 91 day stabilisation period (mean of each measurement)

Sheep #	820	821	844	851	875	877
Clean wool growth (mg / cm^2 / d)	1.270	1.528	1.313	1.202	1.304	1.137
Growth rate of fibre cortex($\mu m^3 \times 10^3$ / h)	11.975	9.637	8.883	12.602	9.792	10.299
Fibre length growth rate (μm / d)	2801.9	2689.1	2192.5	3027.5	3114.1	2739.2
Fibre width (μm)	31.5	28.9	30.7	31.1	27.1	29.6
Follicle density (mm^{-2})	35.7	42.0	47.6	24.7	39.9	33.5
Bulb volume ($\mu m^3 \times 10^5$)	2.03	2.15	1.54	2.38	1.57	1.86
Volume of individual bulb cells (μm^3)	253	133	231	295	243	413
Number of cells per bulb	800	1612	667	807	644	450
Germinative volume of skin ($\mu m^3 \times 10^5$ / mm^2)	72.36	90.31	73.41	58.74	62.55	62.23
No. of cells produced per 4μm section per hour	1.51	4.05	4.39	2.46	1.62	1.98
No. of cells produced per bulb per hour	21.7	63.6	56.0	37.4	19.5	27.8
Turnover time of bulb (h)	36.9	25.4	10.53	21.6	33.1	16.2
Cortical cell length (μm)	90.8	85.1	87.8	93.1	87.6	87.0
Cortical cell width (μm)	6.7	7.2	6.9	6.7	6.0	5.7
Cortical cell volume (μm^3)	1137.7	1216.7	1173.3	1154.6	895.1	786.2
Number of cortical cells produced per hour.	10.53	7.92	7.57	10.91	10.94	13.10
Proportion of bulb cells entering the fibre	0.49	0.12	0.14	0.29	0.56	0.47

Table A3.1.2. Daily feed intake for individual sheep in the finewool and strongwool Merino strains (g/kg liveweight/day) during stabilisation period of Experiment 1.
Feed was offered at approximately 30 g/kg liveweight, which was determined by the liveweight of the animal at the start of the week preceeding the period that the feed was offered.

Week begining	20-Jun	26-Jun	4-Jul	11-Jul	18-Jul	25-Jul	1-Aug	7-Aug	22-Aug	29-Aug	5-Sep
Finewool No.											
0	20.0	31.0	20.7	22.0	21.5	20.5	19.5	22.3	18.1	24.8	24.2
62	15.0	32.0	22.1	23.4	13.4	20.6	17.9	15.5	16.7	23.8	22.6
753	16.0	28.0	20.2	20.1	17.4	18.7	15.4	17.0	18.1	25.6	24.0
1187	30.0	32.0	31.3	28.8	25.9	30.0	29.0	29.0	27.6	29.0	31.0
1237	30.0	31.0	30.0	29.0	21.7	30.0	31.0	27.4	27.0	29.1	28.4
1242	30.0	30.0	30.0	29.1	30.0	29.2	29.2	29.2	30.0	24.4	29.3
Strongwool No.											
820	25.0	27.0	29.0	29.2	26.2	29.1	29.5	29.6	23.8	27.3	23.6
821	30.0	28.0	31.1	31.1	26.5	29.1	27.0	29.1	15.0	28.6	26.1
844	16.0	23.0	29.4	31.8	27.3	25.0	34.7	25.3	23.1	28.5	28.6
851	25.0	30.0	31.2	31.2	26.7	28.9	31.7	27.9	19.4	27.2	30.0
875	24.0	31.0	32.6	21.5	35.0	28.8	31.3	27.7	15.0	27.4	29.5
877	30.0	31.0	29.5	30.5	26.1	28.6	29.1	30.9	23.8	26.4	29.2



A3.1.3a. Number of arrested cells per bulb section after colchicii injection plotted against time in the finewool (a) and strongwool Merinos (b).

A3.1.3b. Correlation coefficients (r) of the regression on the number of arrested cells in the follicle bulb in finewool and strongwool Merinos after colchicine injection over time, with and without the 0 hour sample included in the regression analysis

Finewool Merino	r (0 - 4 h)	r (1 - 4h)
0	0.94	0.99
62	0.95	1.00
753	0.91	0.96
1187	0.93	1.00
1237	0.88	0.99
1242	0.96	1.00
Strongwool Merino		
820	0.97	1.00
821	0.98	0.99
844	0.97	0.99
851	0.97	0.97
875	0.95	0.97
877	0.95	0.97

A3.2.1. Follicle density, bulb volume and total germinative volume of the skin (Germ vol) of the Control and Stud flocks from Experiment 2 (Chapter 2) (Standard error of the mean in parentheses)

Control	Follicle density (mm^{-2})	Bulb volume ($\mu m^3 \times 10^4$)	Germ vol ($\mu m^3 \times 10^5 mm^{-2}$)
1 30	41.0 (2.02)	12.5 (0.82)	51.16
1 39	58.3 (3.08)	10.4 (0.67)	60.73
1 42	34.0 (2.34)	15.3 (0.86)	52.14
2 20	49.7 (3.95)	16.0 (0.65)	79.79
2 37	40.8 (2.01)	17.3 (1.03)	70.43
2 39	64.9 (3.32)	10.1 (0.81)	65.68
3 30	58.0 (1.97)	15.8 (1.48)	91.81
3 37	35.0 (3.38)	17.5 (1.04)	61.25
3 46	33.9 (3.86)	16.9 (0.66)	57.28
4 22	43.2 (2.95)	16.1 (1.07)	69.71
4 32	39.8 (3.55)	16.1 (0.97)	39.76
4 42	38.6 (1.45)	17.6 (0.85)	67.82
Stud			
13	52.9 (2.51)	16.0 (1.38)	84.43
77	38.2 (3.25)	14.3 (0.81)	54.73
78	36.0 (2.66)	15.4 (0.92)	55.27
203	67.9 (2.38)	16.5 (0.80)	112.01
265	62.3 (3.44)	16.9 (0.93)	105.61
352	64.4 (3.27)	13.3 90.89)	85.62
462	51.3 (3.18)	18.1 (0.73)	93.00
538	59.8 (2.17)	14.7 (0.89)	87.86
601	91.8 (3.08)	11.7 (0.74)	107.17
626	39.5 (2.33)	18.9 (0.80)	74.73
633	57.6 (1.82)	18.0 (1.04)	103.67
637	61.3 (4.65)	15.1 (1.06)	92.49

Table A3.2.2a. Daily feed intake for individual sheep from the ANAMA control flock (g/kg liveweight/day) during stabilisation period of Experiment 2.
Note that feed was offered at 30 g/kg bodyweight from 12 December to the 5 January, then the ration was at 24g/kg bodyweight until 13 February after which it was decreased to 22g/kg bodyweight.

Sheep no.	12-Dec	20-Dec	28-Dec	12-Jan	25-Jan	1-Feb	13-Feb	28-Feb
1 30	21.8	19.0	22.3	20.7	17.0	19.8	20.7	21.6
1 39	28.5	32.8	29.8	24.1	25.0	20.7	21.1	21.8
1 42	25.3	10.3	28.9	25.0	23.9	21.3	20.8	20.4
2 20	23.1	28.3	29.8	23.4	24.5	19.3	21.6	22.0
2 37	29.1	28.6	28.8	24.1	25.0	21.3	20.6	21.0
2 39	21.2	23.9	28.1	25.0	17.6	20.5	20.8	17.0
3 30	28.3	28.8	29.6	24.1	25.0	21.8	20.3	21.1
3 37	28.2	25.3	28.6	25.0	18.6	21.4	20.5	21.4
3 46	28.3	29.2	28.3	23.4	24.5	21.2	21.6	20.4
4 22	29.3	28.8	29.0	24.6	24.6	24.6	19.9	21.2
4 32	26.5	21.6	30.0	17.0	24.1	21.4	20.7	21.1
4 42	27.3	28.8	29.0	24.6	25.0	16.7	21.5	21.9

Table A3.2.2b. Daily feed intake for individual sheep from the ANAMA stud flock (g/kg liveweight/day) during stabilisation period of Experiment 2.
Note that feed was offered at 30 g/kg bodyweight from 12 December to the 5 January, then the ration was at 24g/kg bodyweight until 13 February after which it was decreased to 22g/kg bodyweight.

Sheep no.	12-Dec	20-Dec	28-Dec	12-Jan	25-Jan	1-Feb	13-Feb	28-Feb
13	29.7	28.6	28.8	24.2	23.9	22.0	20.3	21.4
77	27.4	29.2	29.3	24.7	21.8	19.9	21.3	21.3
78	28.9	28.6	30.0	25.0	24.4	20.4	21.2	21.2
203	29.0	28.6	29.4	23.9	24.3	21.1	22.2	21.1
265	21.4	22.3	29.6	19.2	21.0	21.2	20.1	17.6
352	28.8	29.0	27.5	25.0	16.6	21.6	21.1	21.1
462	27.1	29.9	30.0	23.8	24.3	21.3	21.1	21.1
538	17.2	26.2	27.1	23.7	24.2	21.9	21.9	21.9
601	29.0	28.6	29.0	23.8	25.0	20.9	21.7	20.6
626	28.6	28.6	28.4	23.9	5.4	21.1	21.0	21.0
633	24.8	26.2	26.7	23.9	16.1	18.9	19.6	16.9
637	26.3	25.4	30.0	22.3	22.8	20.0	16.9	20.8

A4.1. Mean (± s.e.m.) and coefficient of variation of blood flow through the midside and abdominal flank of finewool and strongwool Merinos measured with the laser Doppler velocimeter in Experiments 3 and 4.

	Finewool		Strongwool	
	Mean ± sem	CV (%)	Mean ± sem	CV (%)
Experiment 3	2.6 ± 0.15	20.1	3.5 ± 0.25	25.3
	2.7 ± 0.17	21.4	3.5 ± 0.18	17.2
	2.5 ± 0.09	12.4	3.9 ± 0.18	16.5
	3.2 ± 0.22	23.3	3.7 ± 0.28	26.0
mean ± sem		19.3 ± 2.39		21.3 ± 2.55
Experiment 4	4.6 ± 0.69	36.5	3.1 ± 0.48	38.1
- midside	2.5 ± 0.23	23.2	4.5 ± 0.76	41.9
	3.0 ± 0.19	15.4	2.7 ± 0.57	51.9
	2.5 ± 0.42	40.7	3.6 ± 0.31	20.9
mean ± sem		29.0 ± 5.86		38.2 ± 6.46
Experiment 4	4.7 ± 0.78	40.8	4.8 ± 0.79	40.4
- flank	3.1 ± 0.29	23.1	5.4 ± 0.71	32.4
	3.5 ± 0.41	28.6	3.8 ± 0.96	62.3
	5.3 ± 1.31	60.9	3.2 ± 0.38	28.8
mean ± sem		38.4 ± 8.38		41.0 ± 7.51

A5.1. The pH of the media after three hours of incubation at 37°C (pH), sampling day, the level of ^{3}H-glucose uptake (CPM/mg tissue) and ^{35}S-cysteine uptake (CPM/mg tissue) for each of the blank samples and and skin samples from finewool and strongwool Merinos (mean ± standard error of the mean)

		pH	^{3}H-Glucose	^{35}S-Cysteine
Blank 1	Day 1	8.3	49.6 ± 2.75	27.6 ± 1.55
Blank 2	Day 2	8.5	53.2 ± 4.02	34.7 ± 3.64
Finewool-908	Day 1	7.5	300.2 ± 19.23	125.7 ± 6.77
-985	Day 1	7.5	219.7 ± 15.31	75.8 ± 6.04
-910	Day 2	7.7	301.2 ± 25.11	159.3 ± 12.78
-992	Day 2	7.7	201.8 ± 15.15	100.3 ± 9.77
Strongwool-43	Day 1	7.6	368.0 ± 36.18	150.4 ± 17.58
-86	Day 1	7.6	279.5 ± 23.08	97.4 ± 8.00
-289	Day 2	7.7	502.4 ± 30.20	215.8 ± 14.86
-495	Day 2	7.8	396.4 ± 24.22	155.3 ± 9.39

A6.1.a. Mean (± standard deviation) of characteristics measured in Experiment 6 (Chapter 6) for the determination of glucose and cystine uptake by the skin of finewool Merinos

		86	87	89	90
	Clean wool growth ($mg/cm^2/d$)	0.44	0.47	0.45	0.48
	Follicle density (mm^{-2})	36 ± 5.6	54 ± 17.7	36±10.2	38 ± 7.3
	Bulb volume ($\mu m^3 x10^3$)	166 ± 55.7	145 ± 37.5	141 ± 43.7	152 ± 56.3
	Germinative tissue in the skin ($[\mu m^3/mm^2]x10^5$)	59.99	78.32	51.02	57.53
	Blood flow (ml/min/100g tissue)	2.2 ± 0.70	2.8 ± 0.62	2.4 ± 0.36	1.9 ± 0.15
Mean	Arterial glucose concentration (µg/ml)	546	535	561	535
"	Arterial glucose flow (mg/min/100g tissue)	1.20	1.50	1.35	1.02
"	Venous glucose concentration (µg/ml)	460	637	587	488
"	Arterial cystine concentration (µg/ml)	1.67¥	2.25	1.86¥	1.79¥
"	Arterial cystine flow (µg/min/100g tissue)	3.67	6.30	4.46	3.41
"	Venous cystine concentration (µg/ml)	1.29	1.77¥	3.00	1.71
4 hour	Arterial glucose concentration (µg/ml)	569 ± 32.3	560 ± 22.0	561 ± 54.5	535 ± 31.2
"	Specific radioactivity of glucose (nCi/µg)	4.46	3.29	6.11	4.16
	Blood flow - site 1 (ml/min/100g tissue)	1.2	2.3	1.9	2.0
	- site 2 (ml/min/100g tissue)	2.5	2.5	2.5	2.0
"	Glucose flow -site 1 (mg/min/100g tissue)	0.6	1.3	1.1	1.0
"	- site 2 (mg/min/100g tissue)	1.4	1.4	1.4	1.1
"	Proportion ^3H in skin - site 1	0.68	0.85	0.91	0.51
"	- site 2	0.66	0.76	0.86	0.59
"	Arterial cystine concentration (µg/ml)	1.67	1.75	1.87	1.79
"	Specific radioactivity of cystine (nCi/µg)	0.38	0.33	0.50	0.46
"	Cystine flow -site 1 (µg/min/100g tissue)	2.0	4.0	3.6	3.6
"	- site 2 (µg/min/100g tissue)	4.2	4.4	5.8	3.6
"	Proportion ^{35}S in skin - - site 1	1.46	0.80	1.90	0.89
"	- site 2	1.02	1.45	2.47	0.86

† Mean of 0 and 4 hour samples

¥ 4 hour sample only

A6.1.b. Mean (± standard deviation) of characteristics measured in Experiment 6 (Chapter 6) for the determination of glucose and cystine uptake by the skin of strongwool Merinos

		93	94	95	96
	Clean wool growth ($mg/cm^2/d$)	0.79	0.88	0.71	0.63
	Follicle density (mm^{-2})	42 ± 5.1	43 ± 8.3	62 ± 9.2	56 ± 10.5
	Bulb volume ($\mu m^3 x10^3$)	239 ± 100.0	267 ± 85	239 ± 49.7	226 ± 80.8
	Germinative tissue in the skin ($[\mu m^3/mm^2]x10^5$)	100.43	113.77	148.35	132.70
	Blood flow (ml/min/100g tissue)	4.8¥	6.1 ± 1.75	6.1¥	2.7 ± 2.10
Mean†	Arterial glucose concentration (µg/ml)	562	519	497	526
"	Arterial glucose flow (mg/min/100g tissue)	2.70	3.17	3.03	1.42
"	Venous glucose concentration (µg/ml)	590	512	404	482
"	Arterial cystine concentration (µg/ml)	2.84	1.87¥	1.98	2.61
"	Arterial cystine flow (µg/min/100g tissue)	13.6	11.41	12.08	4.51
"	Venous cystine concentration (µg/ml)	1.84	3.50	2.15§	2.82
4 hour	Arterial glucose concentration (µg/ml)	565 ± 64.5	519 ± 32.5	528 ± 38.8	541 ± 12.8
"	Specific radioactivity of glucose (nCi/µg)	2.36	3.19	-	1.97
	Blood flow - site 1 (ml/min/100g tissue)	3.0	6.3	6.8	1.1
	- site 2 (ml/min/100g tissue)	6.6	8.4	5.4	1.2
"	Glucose flow -site 1 (mg/min/100g tissue)	1.7	3.3	-	0.6
"	- site 2 (mg/min/100g tissue)	3.7	4.4	-	0.6
"	Proportion ^{3}Hin skin - - site 1	1.05	0.66	-	1.47
"	- site 2)	0.94	0.81	-	1.52
"	Arterial cystine concentration (µg/ml)	1.92	1.87	1.72	1.68
"	Specific radioactivity of cystine (nCi/µg)	0.16	0.29	-	0.22
"	Cystine flow -site 1 (µg/min/100g tissue)	5.8	11.8	11.7	1.9
"	- site 2 (µg/min/100g tissue)	12.7	15.7	9.3	2.0
"	Proportion ^{35}S in skin site 1 ( )	2.29	1.77	-	1.47
"	- site 2	1.80	1.74	-	1.52

† Mean of 0 and 4 hour samples

§ 0 hour sample only

¥ 4 hour sample only

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