Aspects Of Wool Follicle Morphology and Cell Proliferation in Romney Sheep Selected For High Fleece Production

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Veterinary Science at Massey University

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This work is dedicated to my parents, and especially to my father, who always encouraged and supported me in my efforts, and who unfortunately did not live to see me completing my work and degree.

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DISCUSSION

ABSTRACT

The mechanisms involved in the expression of genotypic differences in wool production have been investigated. Understanding the control of wool growth involves the understanding of the function of the follicle. The specific objectives were to clarify some of these mechanisms within the wool follicle in this thesis using histological techniques on two lines of New Zealand Romney sheep differing in their level of annual wool production.

A method was established to identify replicating cells in the follicle bulb, by administering the thymidine analogue bromodeoxyuridine (BrdU) to the skin, by either local infusion or intracutaneous injection. Immunocytochemical detection of incorporated BrdU allowed visualization of BrdU labelled, proliferating cells. Various follicle dimensional measurements made using image analysis were tested for their ability to discriminate between morphological variations related to functional changes in the follicle bulb.

The characteristics of the wool fibres of both lines, such as length and diameter, were determined at three stages of the year and subsequently related to results obtained on follicle characteristics. Fibre volume output was increased in sheep with a higher level of wool production, with the diameter being the component contributing most to this increase.

Metabolic measurements (glycogen, SDH) of follicles using histochemical techniques were undertaken to determine, whether they reflect differences in follicle activity at a time during the year, when line differences in wool production were expected to be greatest. Glycogen storage was not associated with energy requirements of the follicle bulb cells. SDH activity was low, suggesting that the follicle utilises glucose mainly by anaerobic glycolysis.

Investigations of individual follicles emphasised follicle dimensions and the proliferating bulb cell population at three stages of the year. Measurements were based upon the use of intracutaneously administered BrdU to assess the replicating cell population in wool follicles. Immunocytochemical detection techniques in association with image analysis enabled quantification of changes in bulb cell replication and follicle dimensions. Both genotypes exhibited a seasonal pattern of follicle changes, with higher values occurring during summer. The higher producing line of sheep showed their advantage by developing larger follicles, larger dermal papillae and larger germinative tissue areas, and therefore larger numbers of proliferating

bulb cells.

Close relationships between follicle diameters and fibre diameters of fibre sections measured within the hair canal and at the surface existed.

The width and area of the fibre cortex and the IRS at a level above the top of the dermal papilla was determined. Proportional changes were observed. This indicates the existence of a redistribution mechanism of cells to cortex or IRS, which is partly influenced by genotype. In sheep genetically inferior in wool production, relatively more bulb cells migrate into the IRS during times of increased bulb cells production (summer) than do cells of sheep on higher production levels.

Theories on the possible influence of the dermal papilla on cell migration in the bulb and on the expression of different follicle components are discussed.

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List of Abbreviations

AOV	- analysis of variance (one-way)
BM	- basement membrane
BrdU	- 5-bromo-2'-deoxyuridine
CLT	- control treatment group
FD	- fibre diameter
FL	- fibre length
FWT	- fleeceweight selected group of sheep
GFW	- greasy fleece weight
GTA	- germinative tissue area
IRS	- inner root sheath
LSMEAN	- least square mean
NS	- not significant
ORS	- outer root sheath
SDH	- succinate dehydrogenase
SEM	- standard error of the mean

Significance levels for statistical tests

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INTRODUCTION

Skin and follicle morphology

This introduction gives a brief description of the morphology of hair-growing skin and the follicle in general. Textbook descriptions are available from such sources as Ham and Cormack (1979), Dellmann and Brown (1981), Fawcett (1986) and Banks (1986). It first emphasises the development and anatomy of the primary follicle, and includes information on the skin of the sheep as well as monogastric mammals, including humans. The distinction between primary and secondary follicles is given later (page: I.22), with respect to the skin of sheep. There is a special emphasis on aspects of follicle morphology that is relevant to the experimental part of this thesis.

General organization of the integument

Because the hair follicle is a derivative of the dermis and epidermis, it is pertinent to begin a discussion of the structure of the follicle with a general discription of the various layers of the skin.

The integument is derived from two germ layers. The outermost layer, the epidermis, develops from embryonic ectoderm, whereas the dermis (corium), underlying the epidermis, is of mesodermal origin. They are separated from each other by a basal membrane. The subcutis (Tela subcutanea), composed of loose connective tissue, links the dermis to underlying structures such as periostium, perichondrium or fascia.

Epidermis

The epidermis is a stratified, squamous epithelium. The cells form distinct layers which vary in amount from region to region. Hairy skin is generally thinner than hairless skin. Figure I.1 shows the organisation and terminology of the different epidermal layers.

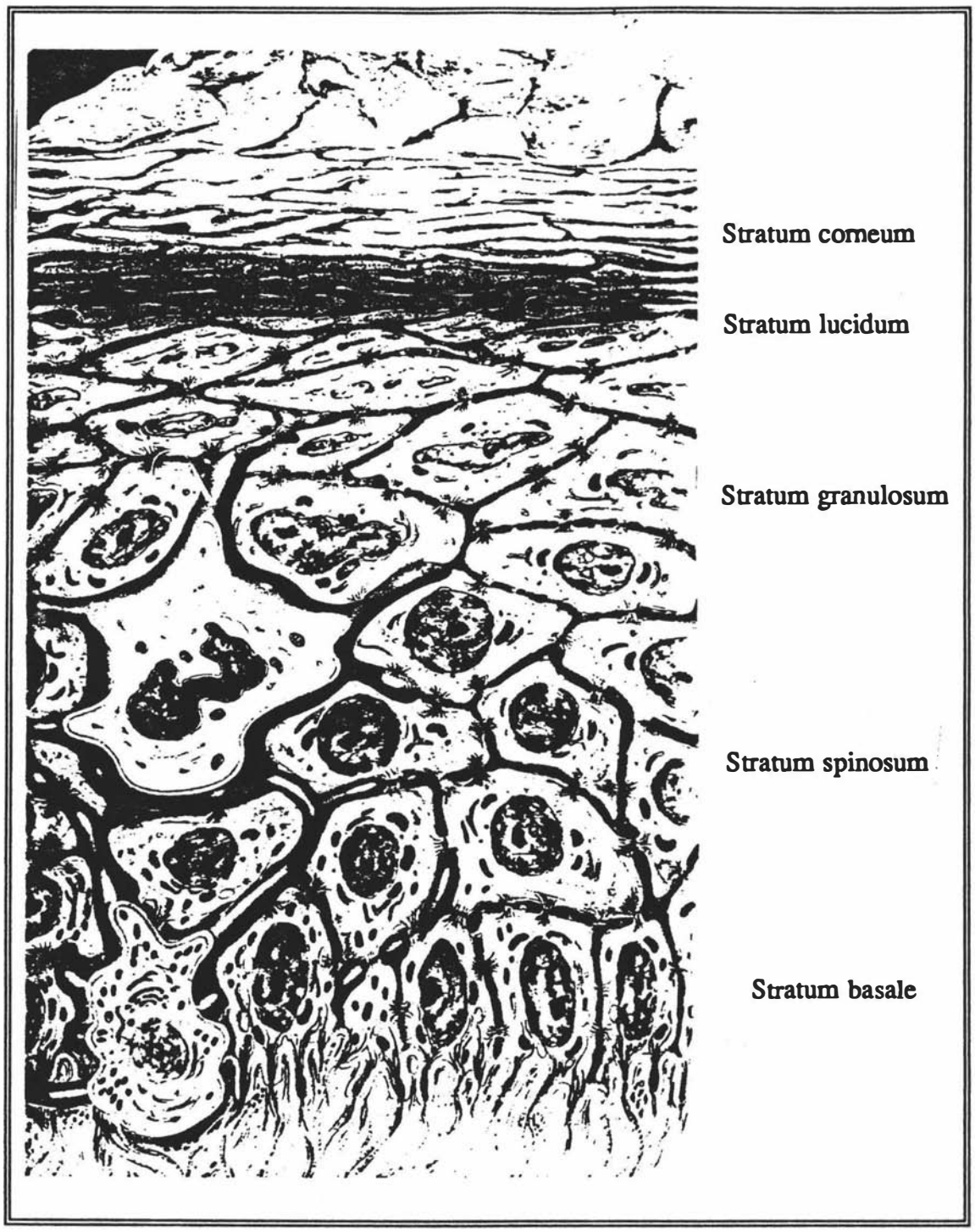

Figure I.1 **Organisation and terminology of the different epidermal layers (Elementa dermatologica, 1987)**

The basal layer (Stratum basale) of cuboidal or columnar cells forms tight connections with the dermis, reaching through the basal membrane by means of hemidesmosomes. The intermediate layers are: 'Stratum spinosum' with intercellular bridges and cells polyhedral to squamous, is usually thin in heavily haired skin; the 'Stratum granulosum' with cells flattened rhomboidal to squamous which can be considered as a layer which shows the onset of keratinization; the 'Stratum lucidum' with cells translucent and squamous, and finally the superficial 'Stratum corneum' which consists of several layers of anucleated squamous, cornified cells. In contrast to the dermis, the epidermis is avascular.

Dermis (Corium)

The dermis is composed of two major connective tissue layers. The papillary layer, underlying the basal membrane, is formed of relatively dense collagen, while deeper in the reticular layer the collagen fibres are loose. Dermal papillae project into the epidermis. By means of these projections, the dermis and epidermis become highly interdigitated and the surface area of the basal membrane is increased, aiding coherence, as well as supplying nutrient and regulating the heat exchange to the epidermis. The dermal papillae contain numerous blood and lymph vessels. Hairy skin is usually not papillated to a great extent. In the reticular layer the blood vessels are generally scarce, forming dense networks only in relation to epidermal appendages that project down into this layer.

Subcutis (Hypodermis) (Tela subcutanea)

The subcutis consists of loose connective tissue which attaches the skin to deep structures. This layer is referred to as 'panniculus adiposus' when infiltrated densely by adipose cells, as is usual in the sheep.

Basement membrane and desmosomes

The immediate support of the epidermis stains readily with the PAS reaction due to the presence of glycosaminoglycans (mucopolysaccharides), Electronmicroscopically, one can distinguish two layers; the deeper one, lamina densa, being denser and therefore the most visible with the light microscope. The lamina densa consists of type IV collagen and several glycosaminoglycans forming a dense network embedded in a homogeneous matrix. Hemidesmosomes enable the tight connection of basal cells to the collagen fibrils of the papillary layer, thus preventing mechanical separation of the two skin layers. Hemidesmosomes are a specialised structure of basal cells, consisting of dense aggregations of cellular tonofibrils which

attach to the cell membrane and contact the structural components of the basal membrane. The superficial part of the basal membrane, the 'lamina vara interna' connects to the basal cell membrane via thin glycoprotein filaments. Beneath the lamina, thin fibrils connect to the collagen network of the dermis. In the epidermis tight cellular connection is achieved through desmosomes (maculae adhaerentes). Desmosomes undergo morphological changes as they pass through the different epidermal layers, until they eventually degenerate to enable sloughing of keratinized cells in the outermost superficial layers of the skin.

The hair follicle

Development of the individual follicle (primary follicle)

Several descriptions of the development of the hair and wool follicle have been published (Ryder and Stephenson, 1968; Michel, 1972; Ryder, 1973; Schnorr, 1985).

Hair follicles can be regarded as modified epidermal structures since they develop in the embryo as epidermal downgrowths into the underlying dermis and subcutis. Also formed are sweat glands and sebaceous glands. The first sign of hair development is a thickening caused by a numerical increase of basal cells in certain genetically predisposed areas of the epidermis. This thickening bulges towards the dermis and causes corresponding cell clusters for the subsequent papilla and follicular connective tissue sheaths to form. The hair germ, so formed, constantly changes shape, growing deeper down into the dermis. Those basal cells which are situated in close approximation to the papilla region show a typical radial orientation, forming the outer sheath of the follicle. The deepest end of the hair germ enlarges and invaginates the developing papilla to finally form the bulb with matrix cells and dermal papilla. Proliferation of matrix cells leads to formation of the first hair which grows upwards towards the skin surface. The hair and the inner root sheath originate from the hair germ. At the same time, the sweat and sebaceous glands associated with the hair, and the insertion for the arrector pili muscle, develop from a localised proliferation of outer root sheath cells. As soon as all different parts of the hair follicle have developed, the actual hair breaks through the skin surface.

General organization of a primary follicle

Since the follicle (dermal papilla and connective tissue sheath excluded) can be regarded as a specialised structure originating from epidermis, its cellular organization follows in general that described for epidermis. The diagram in Figure I.2 illustrates the anatomy of a primary follicle.

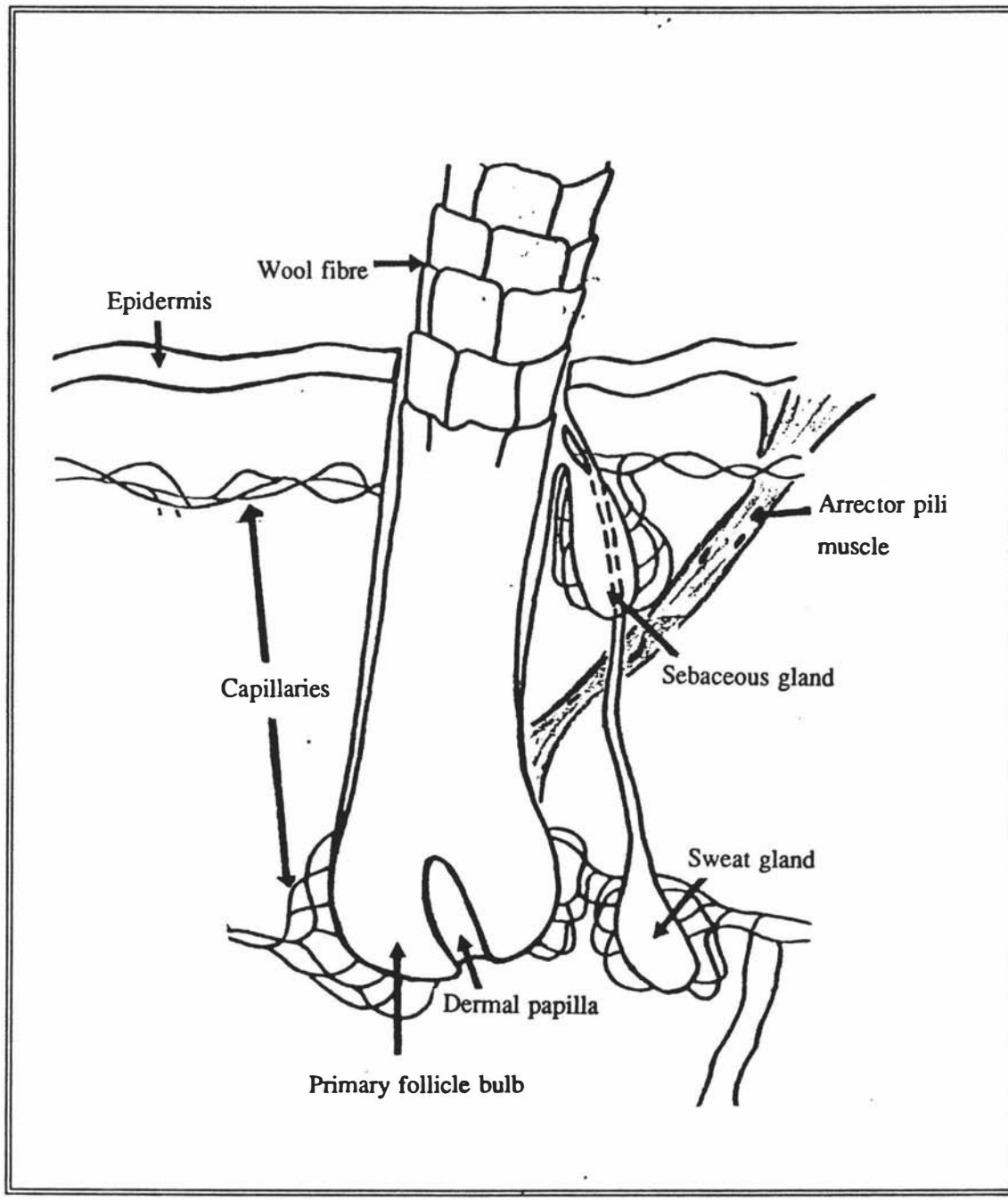

Figure I.2 **General organisation of a primary hair/wool follicle**

The outermost epidermal layer adjacent to the dermal connective tissue sheath is the external or outer root sheath (Vagina epithelialis radicularis externa). It is continuous with the stratum basale, spinosum and granulosum of the epidermis. In the depth of the follicle, it surrounds and becomes continuous with the matrix cells of the bulb. An inner root sheath (Vagina epithelialis radicularis interna) surrounds the actual hair fibre. It consists of three distinct layers, from abaxial to axial: peripheral Henle's layer (Stratum epitheliale externum [pallidum]), intermediate Huxley's layer (Stratum epitheliale internum [granuliferum]) and inner cuticle of the root sheath (Cuticula vaginalis). The inner root sheath (IRS) is not continuous with either the epidermis or the hair. It does not extend beyond the opening of the sebaceous gland into the hair canal. The innermost structure of the follicle, the hair, consists of peripheral cuticle, followed by the cortex and sometimes a central medulla. The inner root sheath and the fibre generate from a cone-shaped epidermal peg, the follicle bulb. It is invaginated at its deepest part by a dermal projection, the dermal papilla.

The (primary) follicle is generally associated with:

- a sebaceous gland, surrounded by the same outer root sheath and connective tissue sheath, opening into the hair canal at the upper third of the follicular shaft.
- a sweat gland, which opens into the hair canal just below skin level
- a smooth muscle (musculus arrector pili) which is attached to the connective tissue sheath of the hair follicle about halfway down the follicle below the sebaceous gland, and the papillary region of the adjacent skin dermis. In response to sympathetic nervous stimulation, its contraction erects the hair.
- a connective tissue sheath (Vagina dermalis radicularis) which is continuous with the dermal papilla and consists of a condensation of collagen fibres arranged in an apparent circular and longitudinal layer (Stratum circulare internum and Stratum longitudinale externum) around the follicle and its associated glands.

Sebaceous glands

In general, at least one sebaceous gland body is associated with one follicle, originating from embryonic outer root sheath cells. These exocrine glands open into the hair canal usually in the upper third of the follicle, immediately above the sloughing zone of the inner root sheath. Their opening ducts are short and wide. The secretion, sebum, is an oily material which lubricates the hair and skin surface. Sebaceous glands are holocrine glands; whole cells become detached and die to form the sebum. Secretory cells are generated from the basal layer of the gland. By proliferation, cells are pushed towards the centre of the gland, synthesising and

accumulating fatty material in their cytoplasm before they become necrotic.

The hair fibre

The hair fibre, the keratinized end product of the follicle, ends in an apex. Its diameter can change repeatedly throughout its length and can vary in the same species with different hair types (Straile, 1965). Nevertheless all hair types have basically the same structure, consisting of the cortex surrounded by a cuticle. Coarser hair fibres possess a central medulla. Figure I.3 shows an example of a medullated and nonmedullated fibre.

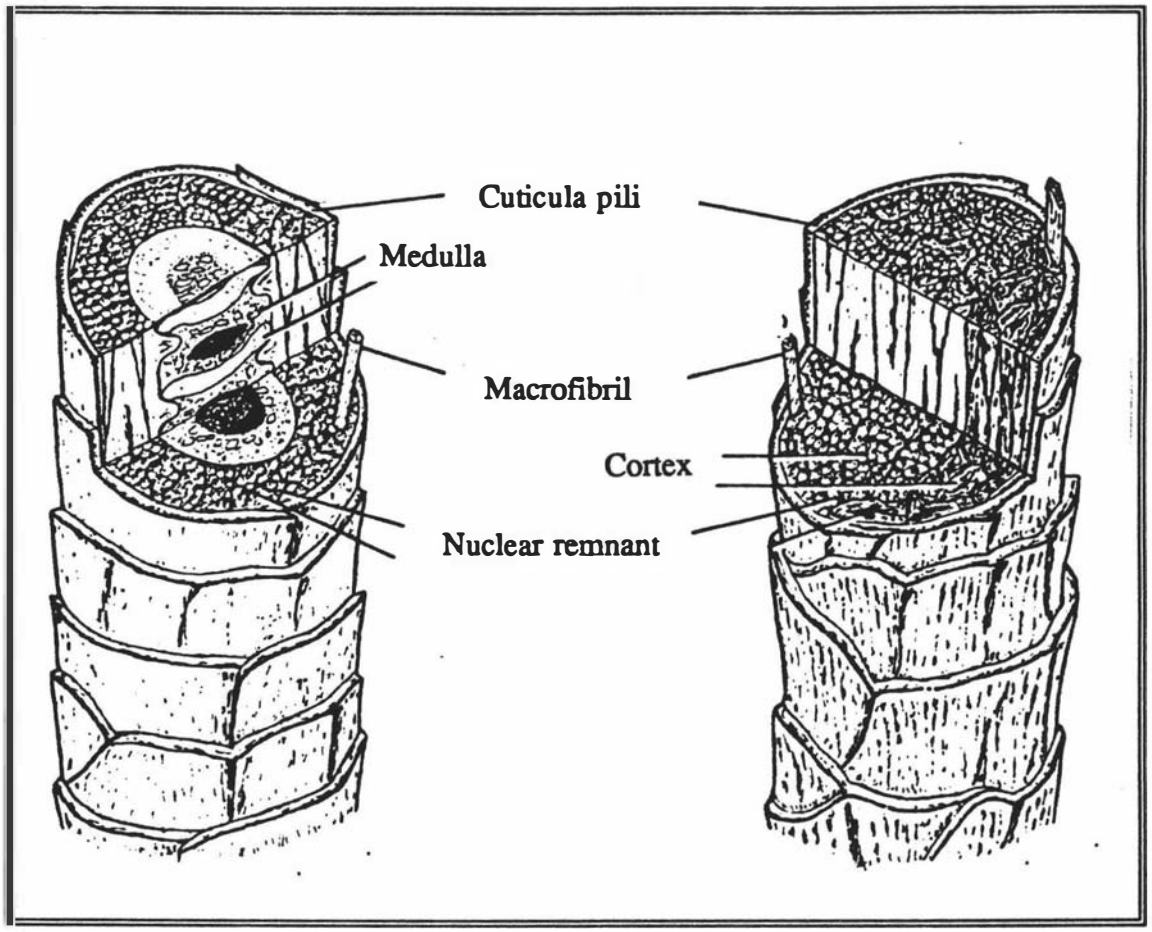

Figure I.3 **Schematic representation of an example of a medullated and nonmedullated fibre (Chapman, 1979)**

Cuticle (Cuticula pili)

The cuticle forms the outermost layer surrounding the whole length of the hair fibre. It consists of flattened cells (0.3-0.5 µm thick) which overlap each other circumferentially and longitudinally in a shingle-like fashion, the free edges of the cells pointing towards the fibre's apex. The overall thickness of the cuticle is determined by the number of cell layers which is in turn a function of the degree of overlapping of these cells. Wool fibres are usually surrounded by one cell layer of cuticle with only a small degree of overlapping of the cell edges (Rogers, 1959), whereas in the mouse one observes five or even more layers (Parakkal, 1969) similar to the situation in human hair (Swift and Bews, 1974). Cuticular cells vary in size and shape between species, being approximately 20 µm long and 30 µm wide in Merino and Lincoln wool fibres. Due to the overlapping of cells, fibres exhibit a specific pattern on their surface which can vary not only between species but also along the length of individual fibres. For the classification and differentiation of mammalian fibres, a special terminology has been established based on light microscopical (Wildman, 1954; Appleyard, 1960) and electron microscopical observations (Bradbury and Leeder, 1970; Schneider, 1972).

Cortex

The cortex is composed of longitudinal cells approximately 100 µm in length. In cross-section they appear polygonal, the central ones 4-5 µm wide, but toward the cuticle they are flatter and rounder at their abaxial surface. Their ends are tapered, often with fingerlike projections to interdigitate with neighbouring cells (Rogers, 1959). In medullated fibres, these projections can be seen branching off abaxially, as well as entering in between the cells of the medulla (Parakkal, 1969). In general, the cortex consists of several different types of cortical cells: in wool fibres there are two segments, namely soft orthocortex and hard paracortex (Mercer, 1953; Fraser and Rogers, 1953). In crimped Merino wool fibres, these two components are found in bilateral organization, the paracortex always running along the inside of the crimp (convex side). In coarser fibres as seen in Lincoln sheep the organization is not as apparent (Fraser and Rogers, 1956). The crimp is associated with an asymmetry of the fibre due to two types of keratin with different physical properties being formed in different regions of the follicle during fibre formation. A third cortical component, designated the metacortex, is found in Southdown- Corriedale and Alpaca fibres (Brown and Onions, 1960). Investigations into the ultrastructure of hair reveals that cortical cells contain filamentous bundles of fibrils (macrofibrils) and remnants of pyknotic nuclei and organelles. These macrofibrils in turn are composed of a microfibril complex which are mainly protein filaments embedded in a matrix

of homogenous non-filament protein acting as cementing material (Birbeck and Mercer, 1957; Fraser et al., 1959). The structural organization of this complex determines in part the difference between orthocortex and paracortex.

Medulla

The medulla is found as a central component in coarse fibres. The cells are largely vacuolated (Auber, 1952). Along the length of a fibre, the medulla can be discontinuous, being interrupted in thinner fibres and continuous in thicker fibres. It is never seen in the apex and the club end (see hair cycle, page: I.24) of the fibre.

The outer root sheath (ORS) (Vagina epithelialis radicularis externa)

Morphology of the ORS

The outer root sheath forms the outermost circumference of epidermal origin of the follicle, separated from the dermis only by the basal membrane (membrana basalis [vitrea]). Descriptions of its morphology and histochemical features are available in the literature mainly for follicles of humans (Montagna, 1956; Montagna and VanScott, 1958; Montagna 1961; Montagna and Ellis 1961; Montagna and Lobitz, 1964; Rogers, 1964) mice (Hardy, 1952; Straile, 1962) and sheep (Auber, 1952; Ryder, 1965). Numerous attempts have been made to divide the outer root sheath (and follicle in general) into several regions along its length, corresponding to morphological, histochemical or functional features. Some examples include division into three layers by Auber (1952), into nine zones by Hardy (1952), into five zones by Straile (1962), into three zones by Chapman (1971) or simply into two regions, the distal region being the permanent and the proximal region being the transient taken from observations of changes during the hair cycle (Priestley, 1967a). It is convenient to first refer to Auber's (1952) system for the description of the outer root sheath (Figure I.4).

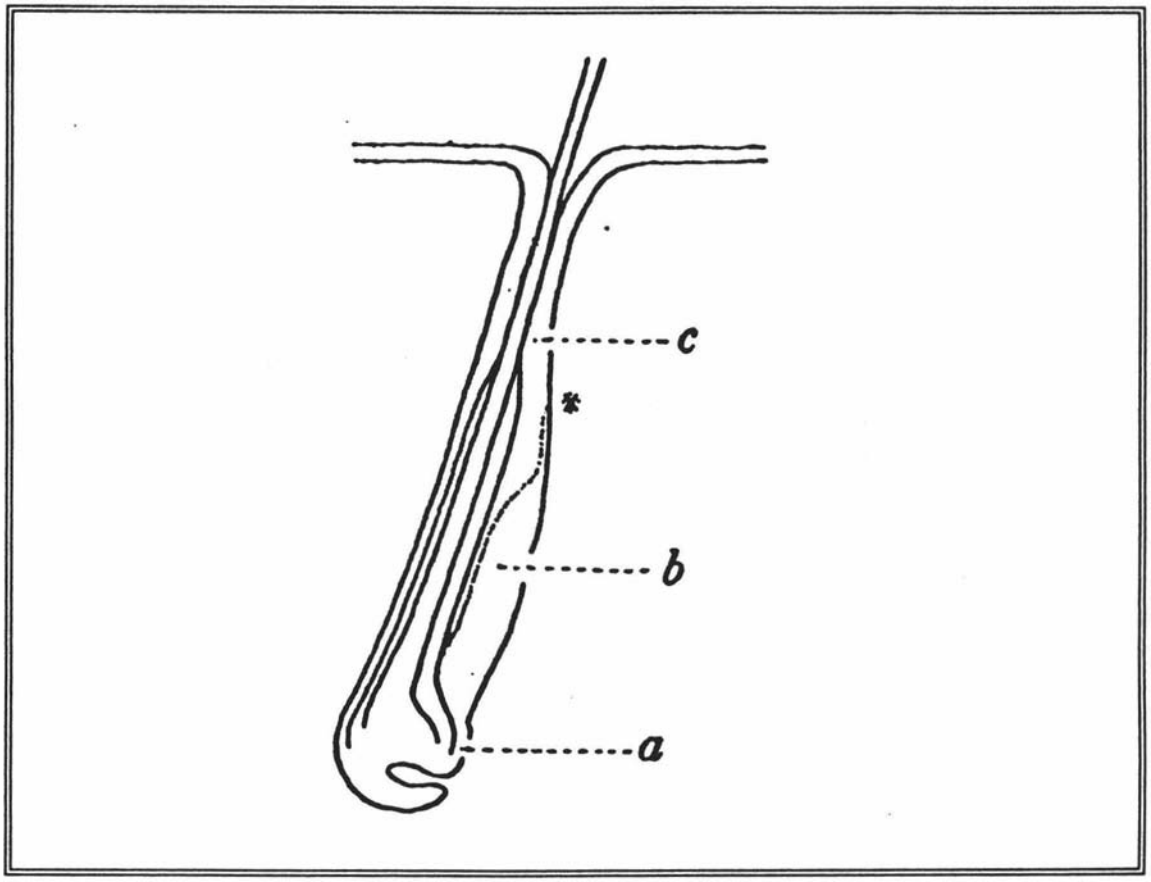

Figure I.4 **Diagram indicating the regions of the ORS (Auber, 1952)**

Zone -a- is the region around the bulb. The ORS cells are elongated and flattened. Some authors (Hardy, 1952; Montagna, 1961; Rogers, 1964; Ryder, 1965) give no further information, whereas others attempt to distinguish a number of cell layers. Montagna (1956) and Montagna and VanScott (1958) describe the existence of two layers of flattened cells, whereas Auber (1952) and Straile (1962) define only one layer. This region coincides with the most peripheral layer formed within the distal bulb region, and the cells do not differ structurally from undifferentiated bulb cells, apart from being flattened. Chapman and Ward (1979) acknowledge the possibility that proliferating bulb cells might give rise to ORS cells surrounding the bulb.

In zone -b- there is accord among observers in so far as the ORS gradually increases in cell layers from three layers just distal to the bulb to a pluristratified layer from that point distally. From this zone till below the sebaceous glands, cells are packed with vacuoles containing glycogen granules (Braun-Falco, 1958; Montagna and VanScott, 1958; Straile, 1962). Furthermore, at this level the cells are rich in mitochondria (Rogers, 1964) as well as ribonucleic acids (RNA) and alkaline phosphatase (in mice) which suggests aerobic metabolism for both

active metabolite transfer to the follicle, and also protein synthesis. Auber (1952) describes a special feature of this zone. The ORS is thickened on the concave side of both ectally and entally (for definition see page: I.22) deflected bulbs in primary follicles (Figure I.4). This feature is seen frequently in sheep since in this species, straight follicles are extremely rare. Montagna (1956) report the same finding for humans especially in the curved follicles of the Negro scalp. However, Montagna and VanScott (1958) describe a contrary phenomenon named "swelling" on the convex side of curved bulbs. An additional "swelling" always occurring on the ental side, is observed by Auber (1952). This sudden change in thickness is assumed to be responsible for the hair having an eccentric position in the follicle and especially in the case of an ectal thickening and an ental swelling, the fibre changing its position twice on its way up the follicle. This thickness change manifests itself mainly in primary follicles in sheep (Auber, 1952) and is particularly evident in large follicles in humans (Montagna, 1956). In region -b- there is general agreement that the ORS cells have an irregular shape, depending on their location in relation to the main fibre axis. In addition to the glycogen storing cells found in this portion, the cells adjacent to the inner root sheath appear slightly flattened (Auber, 1952), up to 1 µm in thickness (Rogers, 1964), with few, if any, vacuoles, intercellular bridges and tonofibrils (Montagna and Ellis, 1956). The most peripherally located cells are tall columnar and are orientated perpendicularly to the main fibre axis. They send minute cytoplasmic processes through the basal membrane (Montagna and VanScott, 1958) which are best developed just distal to the bulb (Montagna, 1956). Distal to the sloughing of the IRS (Auber's zone -c-) the ORS cells are more uniform in width and structure and their cytoplasm appears solid. The structure and organization increasingly resembles that of the epidermis, where the cells are cuboidal and contain no vacuoles.

Theories of cell migration in the ORS

The cytoplasm of ORS cells appears hyalinized and partially keratinized from a level just distal to the bulb (Montagna, 1956; Montagna and VanScott, 1958). From this observation it is suggested that these inner ORS cells might move distally with the inner root sheath (IRS) cells; they are not part of the static ORS structure and the sliding movement of the two sheaths actually occurs between this layer and the rest of the ORS. This view might be supported by the investigations of Straile (1962, 1965) in mice and Chapman (1971) and Chapman et al. (1980) in sheep which have shown that some cell division in the ORS occurs and that cells seem to move distally to be lost at the free surface above the termination of the IRS. These authors leave no doubt as to the fact that the elongated ORS cells adjacent to the IRS migrate distally

with, and at a similar rate to, contemporarily formed IRS cells. These cells harden and slough into the follicular canal distal to IRS cells.

In an extension of these findings and arguments about the relationship of ORS and IRS in zone -b-, Epstein and Maibach (1969) and Chapman et al. (1980) observe inward movement of ORS cells towards the IRS. From studies on the distribution of labelled cells with ^{3}H-thymidine, they draw the conclusion that the ORS is composed of two populations with different proliferation rates and migration patterns. Only Chapman and Ward (1979) acknowledge the possibility that ORS cells can originate from bulb cells.

Throughout the whole length of the ORS, mitotic figures occur infrequently (Chapman, 1971). Appearance of mitotic cells and necrotic cells seem to have a direct relationship, supporting the view the ORS maintains a constant size (Montagna and VanScott, 1958). Auber (1952) describes mitotic figures chiefly in the basal layer.

Basement membrane and connective tissue sheath

Surrounding the ORS is a basement membrane and a connective tissue sheath both of dermal origin (Chapman and Ward, 1979). The basement membrane consists of a basal lamina adjacent to the ORS and two thin layers of collagen fibres, the inner being orientated along the follicle and the outer encircling the follicle (Rogers, 1959; Parakkal, 1969). A continuation of the basement membrane encloses the dermal papilla within the bulb. The connective tissue sheath (Vagina dermalis radicularis) consists of two thicker layers of collagen fibres and fibroblasts. These layers are also arranged at right angles to each other but in reverse order to those of the basal membrane (Stratum circulare internum and Stratum longitudinale externum).

The inner root sheath (IRS) (Vagina epithelialis radicularis interna)

Morphology of the IRS

The inner root sheath, a highly differentiated part of the follicular wall (Auber, 1952), immediately surrounds the hair, Its three components, peripheral Henle's layer (Stratum epitheliale externum [pallidum]), middle Huxley's layer (Stratum epitheliale internum [granuliferum]) and axial cuticular layer (Cuticula vaginalis) originate from proliferating bulb matrix cells (Epitheliocyteus matricis). They terminate distally, proximal to the opening of the sebaceous glands into the follicular canal, in an area which is termed "the sloughing zone of the IRS" (Auber, 1952). This area is characterized morphologically by varying degrees of corrugation in the remaining wall. Before IRS cells start to disintegrate, Henle's and Huxley's layer change their staining properties from basophilic to weak acidophilic, flatten and fuse into

a homogenous single stratum. The cuticular cells retain their individuality but widen their intercellular spaces until only a few cells can be seen over the corrugations. Chemical and enzymatic agents (Auber, 1952) may either cause complete reabsorption of IRS protein, as in sheep, or facilitate mechanical fragmentation, as in man. The corrugations probably serve to increase the surface along which these hypothetical agents can be active.

Auber (1952) divides the IRS into different regions according to phases of maturation, namely:

1) region of differentiation
2) trichohyalin region
3) pre-"keratinization" region
4) "keratinized" region

The boundaries of those regions differ in each layer as well as being located more distally on the side corresponding to the convex side of the bulb deflection. The process of differentiation of the IRS cells is marked by the occurrence of trichohyalin droplets, which appear earliest in the cells of Henle's layer, where they have already become apparent in their cytoplasm at bulb level (Rogers, 1964). In Huxley's layer, trichohyalin droplets do not become present until the distal limit of the follicle bulb, whereas in the cuticular layer they only appear halfway up the follicle shaft.

Maturation of the IRS

The first sign of maturation are the trichohyalin droplets which become apparent in IRS cells. At the same time, filaments less than 10 nm in diameter are found in the cells near trichohyalin droplets (Rogers, 1964). As maturation proceeds, trichohyalin droplets increase in size, both by synthesis of new material and by aggregation with neighbouring droplets. Rogers (1964) states that the electron microscope gives strong evidence that the trichohyalin protein is directly transformed into the filaments through some fundamental configurational changes of the protein, enabling it to reform into a regular structure such as found in the filaments. The filaments finally coalesce to form the rigid structure of the hardened IRS. Auber (1952) observes an abrupt disappearance of trichohyalin through transformation into horn substance in the region he named the 'pre-keratinization region'.

There is some difficulty associated with the terminology connected with the maturation of the IRS. Hardy (1952) states, although it had been previously assumed otherwise, the IRS does not consist of keratin. In the chemical tests she carried out on mouse follicles, IRS cells were resistant to sodium thioglycollate, were digested by pepsin and trypsin, lacked -SH groups

and -S-S- bonds, and had a lower affinity to picric acid. These observations render the view of trichohyalin being a precursor of keratin improbable. Auber's term 'pre-keratinization and keratinized region' in connection with IRS cells needs to be reconsidered.

Trichohyalin exhibits different staining properties to keratin, and is believed to be the major feature distinguishing IRS cells from any other layer in the follicle (Auber, 1952). While travelling up the bulb, cells of Henle's layer change their shape from cuboidal (undifferentiated state) to elongated and reach their maximum length immediately above the bulb where they are about four times as long as wide. Cells of Huxley's layer appear rather thin and compressed until above the bulb equator before they start to widen to a similar diameter as those of Henle's layer. In the trichohyalin region of Huxley's layer, cells send out lateral cytoplasmic processes across the hardened Henle's layer to penetrate as far as the axial layer of the ORS. It is believed that these processes represent living bridges of cytoplasm across the dead Henle's layer to enable nutrients from the ORS to cross over in an axial direction (Montagna and VanScott, 1958). These cells were first described by Hoepke in 1927 and named "Flügelzellen".

Cuticular cells change from a size almost wider than Huxley's in the bulb region to compressed cells with flattened nuclei above the bulb equator. Further up the follicular shaft they convert into a "scaly pattern" by bulging out axially with their proximal cell ends. These cell bulges tend to slightly overlap the distal margin of the cell below (Auber, 1952). The scales formed in this way interlock with the cortical scales of the emerging hair to make a tight connection and hold the hair in its position (Hardy, 1952).

The IRS is in part composed of hard, highly orientated protein which through earlier maturation and hardening may serve to support the very plastic hair cortex in the proximal part of the follicle (Hardy, 1952). In searching for explanations of the earlier hardening of IRS cells, compared to cortical fibre cells, Auber (1952) sees its role in enforcing formative pressure on the fibre. Montagna (1956) observe that because of earlier hardening of the IRS cells, they can easily slide against the partially keratinized axial cells of the ORS with both layers having very smooth surfaces at their interface to facilitate the upward movement. The IRS is believed to move upwards at the same rate as the fibre. Jarrett (1973) regards the IRS as a specialized keratinized structure, distinct from other keratins. He concludes that changes in the IRS have close resemblance to changes that occur in epidermal cells when pressure is applied or when there are benign epidermal invasions of the dermis.

IRS protein is unique in containing RNA, probably attached via the disulphide bonding which is an integral part of the IRS protein molecule. It contains a high level of phospholipids which make it resemble some pathological keratins such as in parakeratosis. Biochemically, IRS

cells differ from hair protein since they do not show any evidence of a Pentose-Phosphate pathway as found in the keratogenous zone of the hair proper, pointing to a rapid cell death in which there is no time for the formation of alternative pathways for carbohydrate metabolism. IRS protein, according to Jarrett (1973), is formed without a granular layer. There is no presence of acid hydrolases, indicating that cells do not undergo destructive hydrolysis like epidermal cells; cell organelles or their constituents remain within the hardened structure. This possibility can be supported by the demonstration of ribosomes in hardened IRS protein. Jarrett concludes with a theory on keratin formation. Pressure effects on the epidermal cells of the inner follicular wall may cause their early death and hardening occurs as a natural sequence, this being different from both the keratin of the superficial epidermis and that of the hair proper.

Variations in IRS thickness

There is contradictory opinion concerning the thickness of the three IRS layers, especially that of Huxley's layer. Montagna (1956) describe three layers, each being one cell thick, but do acknowledge the occurrence of a two cell thick Huxley's layer in large follicles of humans. Auber (1952) states that each layer remains one cell deep throughout its length, but acknowledges an "unusual" thickening of Huxley's and Henle's layer on the concave side of the bulb deflection which persists up to the distal limit of the IRS. Observations made by Priestley (1967a), working with coarse-wooled Herwick sheep, concentrate on the changes occurring in the IRS with seasonal variation in follicular activity. During the summer months, Huxley's layer appears to be one cell thick in all follicles, with the overall shape of the follicle in cross-section being circular. During winter, the active follicles show an asymmetry in their IRS caused by an increase in cell size of both Huxley's and Henle's layer and an additional increase in cell number to two or more cells in thickness in some, mainly primary, follicles. These changes are associated with a change in follicular cross-sectional appearance. The fibre is elliptical in shape and located eccentrically if surrounded by an asymmetrical IRS.

In the hair of the rat ("zig-zag" hair type), Priestley and Rudall (1965) encountered a similar phenomenon. Here Huxley's layer increases asymmetrically in cell size and number at the region of the hair constriction. This observation differs from sheep follicles, in which both Huxley's and Henle's layer change their size. Priestley (1967b) states that the thickening of the IRS is associated with a complementary reduction in fibre diameter. The overall cross-sectional area of the follicle remains both circular and unchanged. Priestley's hypothesis is that a circular cross-section is an essential feature of the follicle. Any marked departure from a circular fibre cross-section is accompanied by changes in the IRS with increase in size and/or number of cells,

especially in Huxley's layer, opposite flattened or indented faces of the fibre. By this mechanism, the total output of cells from the bulb in terms of either cells or cornified material remains unchanged throughout the main stages of fibre growth. By redistribution of cells between fibre and IRS, changes in fibre output are achieved. In contrast, Durward and Rudall (1956) see the asymmetry of the fibre as a result of compression through a thickened IRS. Which is in fact cause or effect has yet to be finally established.

In summary, it is apparent that the IRS can not be regarded as a rigidly developed structure, but can be modified, with changes related to changes in the appearance of the fibre with or without causal sequence. Since both structures originate from the same population of proliferating cells, they should be observed in close connection to each other. Chapters 3 and 4 in this thesis deal with different aspects and theories of the relationship between IRS cells and cortex cells.

The dermal papilla of the follicle

Morphology of the dermal papilla

The dermal papilla can be regarded as a specialized portion of the connective tissue, enclosed by the follicle bulb. It is believed that the papilla influences growth and development of the follicle and fibre, but the degree and method of this influence is still unclear.

The term 'dermal papilla' is used in this thesis to designate the connective tissue element which is enclosed by the follicle bulb during the active growth phase (anagen) and for the compact dermal cells underneath the 'hair germ' during telogen (Montagna, 1956).

The dermal papilla is continuous with the surrounding connective tissue. It contains capillaries of varying density depending on the species, the size of the dermal papilla, and the stage of its activity. The papilla shape is generally pointed at the apex but varying degrees of splitting may occur (Montagna and VanScott, 1958). Striking morphological changes can be observed during the cycle of hair growth, and will be discussed separately (page: I.24).

Functional association with the follicle

Morphological changes in the dermal papilla appear to be closely related to morphological and functional changes in the follicle. Consequently, the dermal papilla has long been recognized as an essential part of the hair. There seem to be constant relationships between the geometrical dimensions of the papilla and various parts of the follicle. Morphology and function of a tissue are often mutually dependent. Although one cannot necessarily deduce physiological from anatomical characteristics, some interferences about physiological relationships of the dermal papilla to the hair follicle on the basis of their anatomical relationship seem reasonable. For example:

1) Auber (1952) acknowledges an influence of papilla dimensions and shape on fibre diameter and the presence and width of the fibre medulla.

2) Burns and Clarkson (1949) found a relationship between blood supply and fibre diameter; the number of blood vessels and papilla size were shown to be positively correlated, as were papilla volume and fibre diameter.

3) Rudall (1956) dissected wool follicles of Romney lambs, and established relationships between the number of mitoses seen in the bulb, the size of the bulb, the papilla volume, the papilla area and the fibre diameter. The rate of wool growth apparently corresponded to the ratio of papilla volume to papilla surface area. Furthermore, in winter the papilla height was depressed to about one third of the height measured during summer.

4) In a variety of pathological (baldness, alopecia areata) as well as normal situations in the scalp of humans, VanScott and Ekel (1958) showed that the number of mitoses in the bulb matrix was proportional to the "activity" of the dermal papilla. As a measure of "activity" they took the papilla volume and the number of cells in the papilla.

Significance of the dermal papilla for fibre production

The relative importance of the dermal papilla for fibre growth as well as formation and maintenance of the entire follicular structure has been studied using transplantation techniques. These studies have emphasized that in order to investigate hair growth, one cannot focus attention merely on the fibre or follicle alone. Fibre production needs to be regarded as a consequence of a complex interplay between the anatomical components of the follicle on one side, and the follicle, its local environment (dermal papilla and connective tissue sheath) and systemic factors on the other. Cohen (1965) showed that epidermis grows down into the dermis over the site of a transplanted papilla, and the formation of a new follicle was initiated. The hair produced from this follicle is of the type of hair grown in that area of the epidermis and not of

the type from which the papilla was taken.

On the other hand, observations in rat vibrissae by Oliver (1969) showed that the removal of the dermal papilla in the anagen follicle had no effect on the follicle; a papilla was formed anew and hair growth continued. These observations lead to the question of what different effects the two tissues, dermis and epidermis, have on each other.

Billingham and Silvers (1965) assume a reciprocal interaction between the dermal papilla, the dermis and the epidermis, where the epidermis becomes organized regionally under the influence of the dermis. This idea is clarified in more detail by Cohen (1965), who described a 'two-stage epigenetic effect' in transplantation studies. The dermis affects the epidermis and this in turn determines the size of the dermal papilla associated with it. The dermis by itself does not regulate the size of the papilla unless the epidermis is present as a mediator. The epidermis causes the papilla to change size until a certain shape is reached according to the characteristics of this area of skin. At this stage the epidermal influence declines. The dermal papilla now determines the number of mitoses in the bulb matrix and hence the rate of growth and perhaps the length and width of the resulting fibre.

Oliver and Jahoda (1989) summarised in vitro studies in which they showed that dermal papilla cells tended to form aggregations, a behaviour similar to the embryonic attributes of papilla anagen cells and fresh papilla implants. Cells from the lower region of the connective tissue sheath behaved similarly. These cells could regenerate new papillae, whereas connective tissue cells from more distal parts behaved like skin fibroblasts. A papilla must be present for hair growth, but cells from the dermal connective tissue sheath appear to be the source of the new papillae. The outer root sheath cells and the connective tissue sheath appear to be the essential interactive components for regeneration to occur, suggesting two major functions of the dermal sheath. First, it acts as a cell reservoir for dermal papilla maintenance and in successive hair cycles. Secondly, in conjunction with the basal membrane, it helps to maintain the outer root sheath as a specialized epidermal variant and a counter inductive influence from the surrounding dermis. Oliver and Jahoda (1989) concluded that the dermal papilla is established as a permanent and stable population of specialized fibroblasts which first appears as a cellular aggregate interacting with the epidermis to ensure follicle development. During the continuous interactions with the follicle, the proximal connective tissue sheath acts as a functional unit retaining its embryonic characteristics throughout the life-time of the follicle.

In conclusion, it becomes apparent that the papilla plays a key role both in fibre production and in the over-all control of the hair growth cycle. It may mediate the influence of systemic factors which can modify the innately permanent pattern of follicle behaviour. Chapters 3 and 4 deal specifically with the role of the dermal papilla in wool production, proliferative events in the follicle bulb and dimensional changes of the follicle bulb.

The follicle bulb

Morphology of the follicle bulb

The enlarged portion of the proximal end of the follicle, the follicle bulb, contains the epithelial cell population for production, differentiation and maturation of cells destined to form the various structures of the follicle, namely the fibre, the IRS and the ORS (Chapman and Ward, 1979). The most proximal part of the bulb, frequently referred to as the lower bulb region, contains the undifferentiated, pluripotential (Montagna and Parakkal, 1974), cell population which, by continuous mitotic divisions adds new cells from below, giving rise to the different cell streams pushing upwards in an orderly fashion, to contribute to either of the structures mentioned above (Montagna, 1956).

Attempts have been made to divide the follicle bulb into various regions according to:

- occurrence of cell proliferation
- morphological cell changes
- ultimate cell function

The exact boundary of the germinative cell population is debated. Auber (1952) established a diameter across the widest part of the papilla, the "critical level" and put its location in relation to the width of the fibre medulla. He did not describe this line as having any association with the boundary of germinative tissue cells, although other authors in later studies often refer to Auber's line to define a boundary. Auber did not tightly define the uppermost boundary of the undifferentiated cell region. He saw it anywhere between the top of the papilla and the widest part of the bulb. Montagna (1956) and Montagna and Ellis (1961) divided the follicle bulb into lower and upper bulb. The lower bulb contains undifferentiated matrix cells, capable of undergoing cell division, whereas the upper bulb is further divided into four regions. Region 1 is the pre-elongation region, the wide portion of the upper bulb, where cells align vertically to each other. Region 2 is the cellular elongation region. Region 3 and 4, distal to the bulb are cortical pre-keratinization and keratogenous zone respectively. Montagna (1956) and Montagna and VanScott (1958) compare the upper bulb to the spinous layer of the epidermis in terms of differentiation and maturation processes. VanScott et al. (1963) quoted Auber's critical level as

reference point for a change in the number of mitoses, with more mitosis occurring proximal of this line (lower bulb).

Chapman and Ward (1979) divided the follicle into 5 zones according to the different functions of the cells contained. They generally referred to the bulb area as the mitotically active zone, reaching around the dermal papilla, with a cone-shaped upper limit extending to the tip of the dermal papilla (Chapman et al., 1980; Orwin and Woods, 1982). This zone coincides with the definition given by Short et al. (1965) for the boundary between proliferative and non-proliferative tissue. Short et al. based the definition on the position of distal mitotic nuclei in the bulb, changes in cell size and the level at which differentiation of the IRS and cuticle cells became apparent. The two latter definitions result in a curved boundary line around the papilla. This is different from Fraser's (1965) definition of a straight line across the first cell layer on top of the dermal papilla, in so far as it might account more accurately for a zone chiefly including mitotically active cells. Fraser's definition includes mitotic as well as differentiating cells. Which of these definitions might discriminate best between functional changes in the bulb will be examined in Chapter 3. Henderson (1965) notes that the follicle bulb has no clearly defined upper limit but it would be commonly accepted that its length is equal to the length of the dermal papilla. Following the germinative zone, the next zone encloses cells which are differentiating to form either fibre cells (cuticle, cortex, medulla) or IRS cells (cuticle, Huxley, Henle). During the course of differentiation cells usually grow in size, lose their mitotic potential, and engage in various degrees of protein synthesis which ultimately leads to their maturation with an irreversible structural commitment.

Cell proliferation in the follicle bulb

The configuration of the cell population responsible for the production of follicle cells is still unknown. Nagorcka and Mooney (1982) followed a suggestion of Potten (1978) for epithelial cells in skin, stating that they arise from a population of stem cells, which are attached to the basal membrane and reside in the basal layer. Epstein and Maibach (1969) observed that DNA is synthesised in cells which are distributed throughout the hair bulb, in which case there would be no well-defined basal layer along the papilla. DNA synthesising cells were also observed above Auber's critical line (Montagna, 1956). The occurrence of mitotic figures is not defined to the basal layer (Auber, 1952). They appear in greater number in the lower half of the bulb (VanScott et al., 1963). Montagna and VanScott (1958) believe that every cell underneath Auber's critical level is mitotically active. Auber's definition of the bulb boundary implies that there are no mitoses above the papilla tip, a suggestion which contrasts with the

concepts describing a proliferative basal cell layer around the papilla. These partly controversial concepts on the distribution of mitotic cells in relation to their location to the basal layer as seen in the skin epithelium could be explained using Potten's (1978) asymmetrical amplifying scheme, where several mitoses (up to three) can occur in cells already committed to form a specific tissue. Auber (1952) noted that cell divisions within the bulb occur all over the undifferentiated region and within the cortical tissue, but no further than where these cells become elongated or enlarged. This definition appears to be then most appropriate since it is based on morphological observations, and is associated with differentiation and maturation processes; functional changes in the cell eventually preclude it from undergoing further mitosis. According to Reinertson (1961), epidermal cells undergo mitosis or start to keratinize depending on their position relative to the source of nourishment.

Cell migration

Cells from the matrix move upward in single rows to the upper bulb where they increase in volume and become elongated vertically (Montagna, 1956). Cells destined to contribute to the formation of the IRS move faster and undergo differentiation and maturation earlier and faster than fibre cells (Downes et al., 1966; Epstein and Maibach, 1969; Chapman, 1971; Chapman and Ward, 1979).

Cells produced in the bulb and migrating out of the bulb are believed to follow strict paths (Epstein and Maibach, 1969) or cell streams (Bullough and Laurence, 1958), with their differentiation into specific cell types being predetermined according to their position in the bulb (Chapman and Gemmell, 1971). Cells produced more proximally migrate faster than contemporarily formed cells closer to the dermal papilla apex. This mechanism results in cells of the same generation being transported up the bulb in one line (Nagorcka and Mooney, 1982). Montagna and Parakkal (1974) observed that mitotically active cells are capable of differentiating into any of the epidermal cell types present in the follicle. The different parts of the IRS and the fibre are believed to arise as separate streams of cells in the lower follicle bulb (Ryder, 1965). Auber (1952) observed a zonal arrangement in an axio-abaxial direction, corresponding to cell-streams. Hence, the layers of IRS and fibre arise within the bulb by radial pressure. Orwin and Woods (1982) described intercellular bridges between cells of the same cell line as well as, occasionally, between cell lines in the bulb area. In the upper bulb however, these bridges disappeared, thus allowing for possible movement between cells during early stages of differentiation. While passing from the lower bulb into the follicle shaft, each cell line decreased in cell number (Orwin and Woods, 1982). The presence of intercellular bridges in the lower follicle

indicate that here, cells are closely connected to each other, and hence are forced to move together. Further distally, these connections are lost, so that cells can move independently. This morphological change appears to be functionally significant, since a cell number reduction takes place in the upper bulb. If a number reduction takes place, a change in migration velocity of individual cells must have occurred. If intercellular bridges were retained, cell reduction could only be achieved by cell death. Cell proliferation in the lower bulb would then be inefficient.

Mechanisms altering kinetics and proliferation of epithelial cell populations

Considering the hair follicle as a specialized structure of epithelial origin, it is justifiable to investigate events in the epidermis and relate them to the hair follicle. Studies on human epidermis both in physiological and pathological conditions suggest that the size of the germinative cell population influences the overall mitotic activity of a cell population (VanScott, 1965). Schinckel (1961) linked wool production and the number of germinative cells rather than their turnover time in wool follicles. In normal epidermis, mitoses occur in a single basal cell layer, randomly distributed (VanScott, 1965). In squamous mucosa as well as in pathological conditions such as psoriasis and benign epidermal hyperplasia, higher cell proliferation and somewhat higher turnover and transit times are associated with the enlargement of the dermal papillae (VanScott and Ekel, 1963). The consequent elongation of the dermal-epidermal line causes an increase in the population of germinative cells, which now occupies three layers. In the same way that an enlargement of the subepidermal papillae is needed to support an increased germinal cell population in these skin conditions, the size of the germinal hair matrix population might be controlled by the size of the dermal papilla. When the organization of the epidermis is lost, as during regeneration with transient hyperplasia (Potten, 1978), deep follicle-like structures and folded basement membranes are found, which would increase cell production. Thus, the follicle as an epidermal downgrowth, with a dermal papilla deeply encased into the proliferative matrix, might represent an adaptation for intensified proliferation.

Arrangement of the follicle complex in sheep

It is not sufficient to study isolated follicles. The relationship of individual follicles within their groups is equally pertinent to studies of follicle production. Sheep have two distinct types of follicles named primary and secondary follicles. This heterogeneity is a feature common to all breeds of sheep independent of the type of fleece which they grow (Fraser and Short, 1960). Primary follicles are the largest and are generally associated with sebaceous and

sweat glands and an arrector muscle, whereas secondaries are only associated with sebaceous glands (Carter, 1955). The follicle group, which usually comprises three primary follicles, a trio group, and their associated secondary follicles, is the basic unit, the follicle complex. Figure M.1 (page: M.6) is a photomicrograph of a follicle group in cross-section stained with Sacpic stain, illustrating the features associated with secondary and primary follicles.

Development

The complete follicle population has four phases of maturation:

- 1. central primary follicle
- 2. lateral primary follicles
- 3. prenatal wave of secondary follicle maturation
- 4. postnatal wave of secondary follicle maturation

The initiation of the first follicles starts from around day 50 of gestation. The central primary follicles are the first to be seen, followed by the lateral primary follicles. One central and two lateral primaries form the trio-group, the essential foundation of the follicle complex. From day 85 of gestation, the initiation of secondary follicles commences between the central and lateral primaries (Carter, 1955). The primary follicles lie in distinct rows across the skin with the arrector pili muscles and sweat glands on one side (the ectal side) and the secondary follicles on the opposite (ental) side (Ryder, 1973). All secondary follicles are initiated before birth, but they might not commence fibre production until several weeks after birth (Schinckel, 1953; Fraser, 1953; Fraser, 1954). Secondary follicles are generally smaller, shorter and finer than primaries, the size difference being reflected in the hair grown by these follicles. The later secondaries appear between the first secondaries and the primaries. Occasionally, secondaries increase their number by branching, which seems to be important in fine-woolled Merinos (Hardy and Lyne, 1956). From the developmental point of view, there should be a distinction between the original secondaries arising directly from the epidermis, and the secondary follicles that have formed by branching (derived secondary follicles). The maximum number of follicles that a lamb can form is determined genetically but untoward environmental effects can depress the expression of the full hereditary potential for wool production (Ryder, 1973). Once the follicle population is completed, it is not usual for any more new follicles to be developed. Only occasionally, following injury, small numbers might form anew, as an epidermal downgrowth as well as by branching of follicles around the margin of the wound (Ryder, 1973).

The seasonality of wool production in sheep

In domestic breeds of sheep, seasonal moulting or shedding of the fleece has largely been genetically eliminated, but an annual rhythm of wool production remains, considered to be a remnant of the former mechanism of shedding (Hutchinson, 1965). In some unimproved breeds of sheep, photoperiod induced changes in wool growth rates are visible in fleece growth patterns and shedding cycles, as on the legs of Limousin sheep (Rougeot, 1961), in Soay sheep (Ryder, 1971; Ryder and Lincoln, 1976), in Wiltshire Horn sheep (Slee, 1965; Ryder, 1969) and in Mouflon sheep (Ryder, 1973). Primitive patterns of shedding of hair remains on the legs of Merinos and Southdowns (Hutchinson, 1965).

The variation in annual growth rates of the fleece is considered to be primarily determined by photoperiod (Morris, 1961; Bennett et al. 1962; Hart et al. 1963; Hutchinson, 1965; Ryder and Stephenson, 1968; Hutchinson, 1976) temperature playing a lesser role (Bennett et al. 1962; Lincoln et al., 1980).

In improved breeds of sheep, wool fibres grow continuously, fibre shedding being negligible in Merino and Merino x Southdown crosses (Slee and Carter, 1961; Lyne, 1961). Thus, the seasonal pattern of wool growth in the Merino is considered to be independent of light changes but mainly dependent on nutritional influences (Doney, 1966; Hutchinson, 1976).

In the Romney Marsh (Morris, 1961) and Southdown (Bennett et al. 1962; Hart et al., 1963; Wildman, 1957) photoperiod has been observed to be the principal environmental factor determining the annual rhythm in the rate of wool growth. Story and Ross (1960), Sumner and Wickham (1969) and Bigham et al. (1978) described the seasonal patterns of wool growth in the NZ Romney sheep. Growth is faster in summer. There is also an interaction of season and responsiveness of wool growth to nutrient intake (Sumner, 1979), accentuating seasonal differences (Hawker et al. 1984) due to variations in the partition of nutrients to the follicle (Hawker and Crosbie, 1985).

The hair cycle

Morphological and biochemical features of follicular components during the growth of a follicle are characterized by cyclic periods of activity followed by quiescent periods. This occurs in all mammals, including humans and sheep.

Dry (1926) was the first to classify the stages of hair cycle, with three distinctive periods. The length of the growth period is termed *ANAGEN*, the quiescent stage *TELOGEN* and the short period of follicle regression between these two, *CATAGEN*.

The morphology of the growing anagen follicle follows the description for the primary follicle

given earlier in the Introduction (page: I.4). Only a description of catagen and telogen follicles need to be given here.

Catagen

This is the short period during which the follicle changes from the active to the quiescent stage, and is characterized by striking morphological and functional changes. The active follicle anchored deep in the dermis decreases about one third in length and forms a new end, the club. Epithelial and connective tissue elements of the follicle undergo structural changes toward the quiescent stage. Melanogenesis (in the case of coloured hair) and mitotic activity in the follicle bulb decreases till they finally stop. After the formation of cuticular and matrix cells has ceased, the follicle bulb consists only of cortex cells. As a last step, "club" cells are formed which connect, by numerous desmosomes on their club-like ends, with cortical cells on one side and hair germ cells on the other. Thus, the catagen hair is held in place. The germ cells surrounding the club are generated from the ORS. In its middle part, the ORS consists of several layers, turning into germ cells. Prior to this, they contain numerous autophagovacuoles, groups of mitochondria, ribosomes, endoplasmatic reticulum and glycogen. Plasma membranes develop desmosomes, to interconnect neighbouring cells as well as the dermis. The germ cells form 2-3 layers around the club. After the formation of the club and germ cells, the more proximal located cells become resorbed. These cells start complete disintegration, leading to the asymmetrical shrinkage of the follicle (Chase, 1954).

The club cells are now believed to form from cortical cells, since the filaments they contain appear to hold a certain resemblance (dimension and structure) with filaments in cortical cells. They are, however, not organized to the degree found in keratin structures of cortex and epidermis cells (Brody, 1960).

Several hypotheses exist on the nature of the events leading to a quiescent follicle. Chase (1954) and Ellis and Moretti (1959) regard these as form of degeneration whereas Straile et al. (1961) and Straile (1962) see them as a logical consequence of differentiation of hair bulb cells which remain after cessation of mitotic activity. Kligman (1959) reports that the follicle in catagen undergoes degeneration as well as differentiation of cells.

The dermal papilla and basement membrane in catagen

In the dermal papilla, lysis and resorption of capillary endothelial cells become apparent during catagen (Parakkal, 1966). Histochemical investigations have shown that blood flow through the capillaries adjacent to the bulb decreases in catagen and increases in anagen (Ellis and Moretti, 1959; Chase, 1965). The basement membrane is thin in anagen and telogen. In

catagen, the basement membrane thickens and commences to fold, encasing the atrophied structures of the lower follicle during the late stages of catagen. In the upper follicle, the basement membrane does not change since the cells of the follicle do not change. Finally, the basement membrane is resorbed, probably by perifollicular macrophages. The connective tissue sheath is synthesised and resorbed with every tissue build-up and break-down.

Telogen

In telogen, the follicle reaches a stable state with the hair fibre being anchored in it by means of the club, which sends small keratinized projections into the encasing germ cells. Structurally, the telogen follicle differs greatly from the anagen follicle. The dermal papilla has shrunk to a small cell cluster under the lower end of the follicle. Dermal papilla cells have a large nucleus and little cytoplasm. The telogen follicle lacks the matrix, inner and outer root sheaths and hair cuticle cells found in anagen follicles. In follicles where telogen has been chemically induced, a long peg of ORS cells connecting the dermal papilla and the club are often seen. In sheep, this is observed after administration of glucocorticoid (Chapman and Barrett, 1970), or zyclosphosphoramid (Reis and Chapman, 1974) or mimosine (Reis et al., 1975). The form of the fibre end varies according to the drug used.

Anagen

In anagen, cells regenerate from germ cells in a process similar to the embryonic pattern of follicle development (Pinkus, 1958). More recent studies (Parakkal and Alexander, 1972; Cotsarelis et al., 1990) have revealed that in the mouse, the matrix cells for new hair cycle are derived from a group of late replicating ORS cells below the sebaceous glands.

The Massey University fleeceweight selection flock

The animals used throughout the entire study, with the exception of the study reported in Chapter 2, originated from the Massey University Department of Animal Science selection flocks. This Section briefly describes the history of this flock.

Management and selection procedures for these lines have been described by Blair et al. (1984, 1985). Separate flocks selected against face-cover and for high fleeceweight, as well as a control flock, were derived from the same base flock of New Zealand Romney sheep. Animals were allocated to these three groups at random. The lines were first established at Massey University in 1956. They were closed in 1958 and have been since maintained at 80 ewes. Four 18-month-old rams have been used for mating each year. Replacements each year are chosen

at random for the control line. For the high fleeceweight line, they are based on selection at hogget shearing (14 months of age). The face-cover selection line (Blair et al., 1984) was not used in this study. The selection response to greasy fleeceweight was seen in both ewes and rams with a rate of approximately 0.2 kg wool per year (Blair et al., 1985) with no significant decline in response in later years (Matthew, 1991). After 27 years of single-trait selection in the fleeceweight line, ewe greasy fleece-weight had increased by about 1 kg and average lifetime wool production by approximately 20% relative to the control line (Blair, 1981). The production of greasy wool was 25% greater in fleeceweight sheep than in control sheep with wool growth showing a marked difference in its seasonal pattern (McClelland et al., 1987b) (Figure I.5)

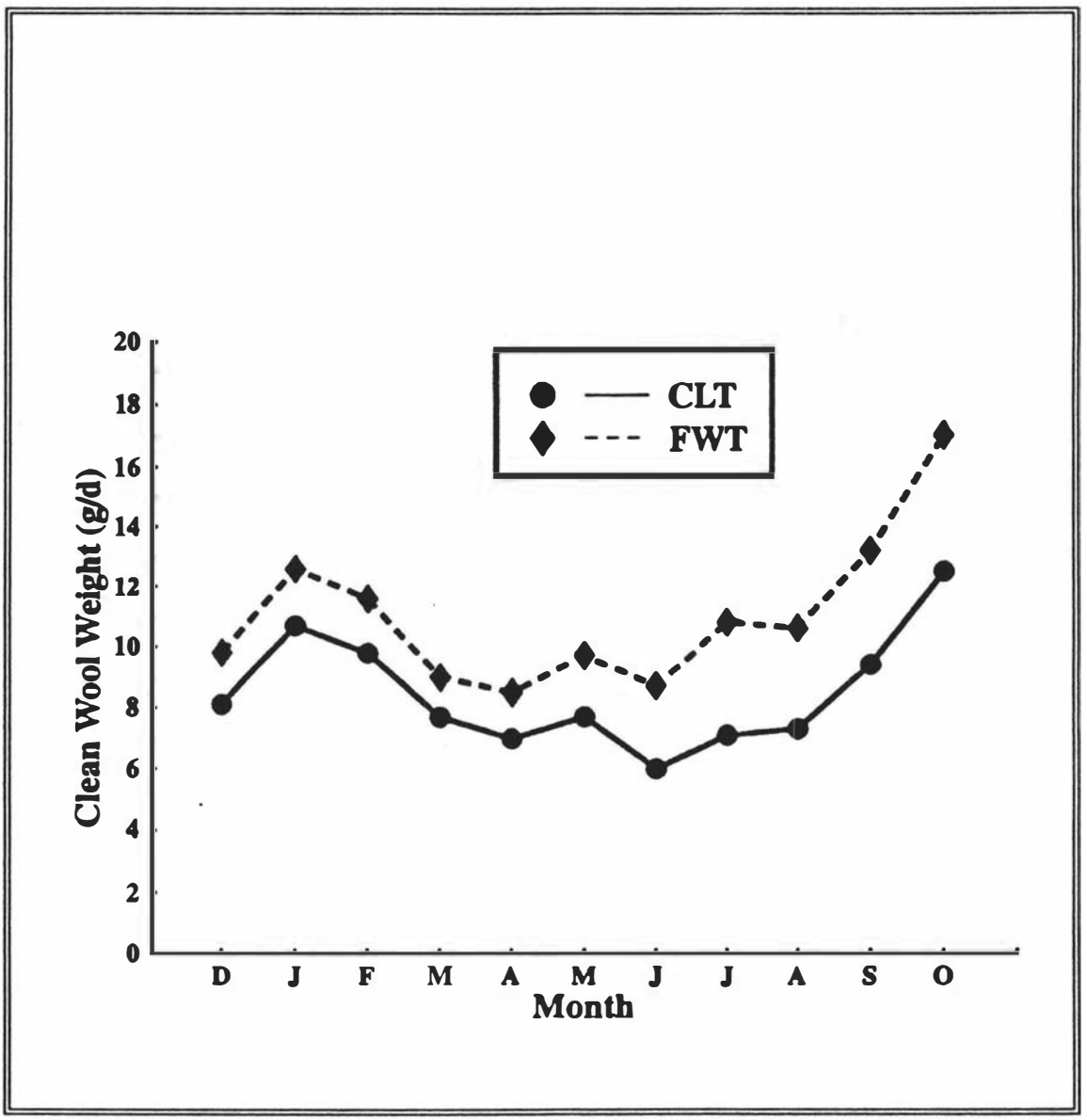

Figure I.5 **Clean wool production (g/day) of fleeceweight (FWT) and control (CLT) sheep (McClelland et al., 1987b)**

During the course of establishing the underlying biological mechanism for the improved productivity in fleeceweight sheep, various experiments have been conducted. Blair et al. (1985) recorded several traits in adult ewes and yearlings which increased in the selection lines: clean wool weight, staple length, mean fibre diameter and liveweight. Variations in food intake did not account for differences in wool growth (McClelland et al., 1987a). It appeared that selection for increased fleece weight had not changed the animal's ability to digest nutrients. Physiological differences between the lines have also been studied (McCutcheon et al., 1987; Clarke et al., 1989; Matthew, 1991) as well as wool sulphur-concentration and output (Antram et al., 1991) and wool sulphur contents at different positions over the body for the same lines (Sun et al., 1991). Fleeceweight rams maintained a lower plasma urea concentration and lower creatinine concentrations than control rams. Differences in plasma creatinine concentration could be due to variations in glomerular filtration rate. Differences in urea concentration could have reflected the differences in glomerular filtration rate and differences in the efficiency of using amino acids for wool production. The wool-sulphur concentration was significantly lower in FWT but the total sulphur output was significantly higher. FWT animals had lower wool sulphur concentrations than controls at all positions measured, suggesting that selection for FWT had reduced wool sulphur content over the entire body. FWT sheep show lower plasma thyroxine (T^4) levels, and, in spring, higher prolactin levels. These two hormones may have a role in the seasonal regulation of wool production. Liu (1991) studied the components of fleeceweight influenced by selection on ewe hoggets. These are greasy fleece weight (GFW), clean fleece weight, clean scoured yield, quality number, total crimp number, staple length, fibre length (FL), mean fibre diameter (FD), body weight, number of primary and secondary follicles per unit area of skin and the S:P ratio. Fibre length accounted for most of the differences in fleece weight between the two lines, whereas mean fibre cross sectional area was the most important contributor to within control flock differences. Also, significant differences were found in fibre cross sectional area and number of fibres per unit area of skin but no differences in smooth body surface area.

In conclusion, the present state of information on these lines does not offer a complete explanation as to how fleeceweight selected sheep maintain a higher average annual wool production. No information on the morphology of wool follicles and related cellular events in the follicle bulb responsible for differences in fibre production is available.

Aims and organisation of the study

Because of the sheep's role as a domesticated wool producing animal, much effort has been made to understand wool growth. To this published literature should be added that dealing with the various aspects of hair growth. Nevertheless, many underlying aspects of both hair and wool growth remain poorly understood. This study has used histological techniques to clarify some morphological mechanisms of growth within the wool follicle.

Two lines of New Zealand Romney sheep in which the line difference was expressed through genetically varying levels of annual wool production (page: I.26), were considered suitable subjects to study genetic effects on the wool follicle.

Different parts of this study address:

1. The characteristics of fibres grown by FWT and CLT sheep, such as length and diameter, including new techniques to measure these traits.
2. The composition and dynamics of the follicle population, including seasonal variations in follicle population activity and follicle density.
3. Possible changes in the metabolic state of the follicles to the time of the year when wool production is believed to have declined most and differences between FWT and CLT sheep are most pronounced.
4. Growth mechanisms of the individual follicle on a cellular level. Major emphasis is put on the germinative region of the follicle bulb, follicle bulb dimensions and proliferating bulb cell population, as well as follicle bulb and dermal papilla interactions.
5. New methods to facilitate proliferation studies using histological techniques as well as exploiting computer techniques to aid in the collection and processing of data.

This thesis includes four chapters investigating the different aspects outlined above. Chapters 1 and 4 deal specifically with histological features of follicles from FWT and CLT sheep. Chapters 2 and 3 determine which are the techniques that best discriminate between FWT and CLT sheep, in terms of cell proliferation and dimensional measurements of the follicle bulb.

Sections of this thesis have already been published or accepted for publication:

Holle,S.A.and Birtles,M.J.; An immunocytochemical method for studying patterns of cell proliferation in the wool follicle. New Zealand Veterinary Journal, 38, 89-93, 1990.

Holle,S.A.; Birtles,M.J.,Westlake,V.J.; In vivo labelling with bromodeoxyuridine for studying cell proliferation in sheep. (Poster presentation). Combined Meeting, Physiological Society of NZ, Australian Neuroscience Society, Australian Physiological and Pharmacological Society, Anatomical Society of Australia and NZ, University of Otago, 28-31.January 1991

Holle,S.A.;Harris, P.M.,;Davies,A.S.; Investigations of wool follicle morphology and cell proliferation in sheep with different levels of wool production. NZ Society of Animal Production, Proceedings (1992), in press.

Holle,S.A. and Harris,P.M.; Studies on the kinetics of in vivo labelling of proliferating follicle bulb cells with 5-bromo-2'-deoxyuridine (BrdU). Australian journal of agricultural research, 1992 in preparation

MATERIALS AND METHODS

Animals

Adult, two-tooth eighteen month old New Zealand Romney rams from both fleeceweight (FWT) and control (CLT) flocks, were used for experiments described in Chapters 1 and 4. Adult New Zealand Romney ewes were used for the experiment in Chapter 2.

Animal management procedures

Indoors

For the experiment in Chapter 1, rams were brought indoors and housed in individual pens. Special details of their maintenance are given in this chapter.

On Pasture

The sheep (rams and ewes) for the experiments described in Chapters 2 and 4 grazed pasture dominated by white clover, ryegrass and lucerne. They were drenched with anthelmintic (Ivomec; Merck, Sharpe and Dohme) before the start of the experimental period. The health status of these animals was satisfactory throughout the time of the experiment. Body weights were recorded before and during the experimental period.

Experimental Period

The experiments described in Chapter 1 was conducted in a period from winter until early spring (June until October). The experiment in Chapter 4 was conducted from June until December, representing a period from winter until early summer.

Collection of skin samples

For subsequent paraffin wax embedding, skin samples were taken with a 1 cm diameter trephine. Tissues were directly transferred into Bouin's fluid fixative (Culling, 1974) where they remained for a maximum of 12 hours before being changed into 70% ethanol for further storage. For histochemical procedures, some skin samples were collected using the freeze-clamping

technique. A tissue clamp was cooled in liquid nitrogen to -169°C, and used to clamp an oval piece of skin approximately 0.5 x 1.5 cm. This tissue piece was excised and immediately transferred into iso-pentane (2-methylbutane, Analar, BDH Limited, Poole, England) cooled in liquid nitrogen. This technique does not cause pain to the animal since the skin is anaesthetized by the cold. The tissue had to be frozen rapidly to minimize freezing artifacts with resultant damage to tissue and cell components. Tissues could not be transferred into liquid nitrogen directly, but into precooled iso-pentane because it rapidly conducts heat from the tissue. The samples were removed from the iso-pentane and immediately mounted on pre-cooled stainless steel specimen holders with OCT-compound (Tissue-Tek, Miles Scientific, Division of Miles Laboratories, Inc, Naperville, Illinois) and sections 12 µm thick were cut using a cryostat microtome (Lipshaw Electric Cryotome, Lipshaw Manufacturing Co., Detroit, Michigan, USA). Sections were attached to clean dry slides and stored at -20°C prior to histochemical procedures.

Collection of fibre samples

All fibre samples were taken using clippers (Sunbeam Animal Clippers, Sunbeam Co Ltd, NSW, Australia) with a No. 40 blade. This ensures that 1.5 mm of fibre stays on the animal and has to be taken into consideration when measuring fibre length or determining clean wool weight (Langlands and Wheeler, 1968). For total wool weight measurement, the animals were shorn and the wool weighed directly afterwards.

Dye-banding procedure

All rams used throughout the experiment, whether kept indoors or outdoors, had a strip of wool marked on either side on the mid-side area (Chapman and Wheeler, 1963). The method uses an aqueous solution of Durafur Black R (Imperial Chemical Industries Ltd.) to dye bands of greasy wool, by directly applying the dye solution to the fibres at skin level from a fine tipped Pasteur pipette. The solution was prepared immediately prior to use by dissolving 0.8% (w/v) of Durafur Black R flakes in cold water and adding 0.8% (v/v) of concentrated hydrogen peroxide (100 vol) to it as an oxidant. When applied to the skin, this solution produces a permanent brown-black coloration in the wool fibres which is not affected by climatic conditions.

Histological techniques

Chemicals and stains

All chemicals and stains used in histological processing and staining are listed in the Appendix (page: A.1 to A.4).

Tissue processing and microtomy

Except for histochemical techniques, all samples were processed for paraffin wax embedding. Tissues were dehydrated through a series of graded ethanols, cleared in three changes of xylene and infiltrated with paraffin wax at 56°C according to a standard schedule on an automatic tissue processor (Shandon Elliot, London) shown in Table A.1 in the Appendix. The samples were then embedded on a Tissue-Tek embedding console (Tissue-Tek, Miles Scientific). Sections were cut using a base sledge microtome (Leitz, Wetzlar, Germany) or a Supercut 2050 microtome (Reichard-Jung GmbH, Nussloch, Germany) floated on warm water and mounted on slides previously cleaned in ethanol and coated with poly-L-lysine (Sigma) to prevent subsequent section detachment. For immunocytochemical detection of incorporated BrdU, sections were cut 5 µm in thickness parallel to the long axis of the wool follicles. For all other histological staining techniques, section thickness was 8 µm cut parallel to the skin surface. For histochemical techniques, a cryostat was used to section the tissues parallel to the long axis of the fibres at a thickness of 12 µm. Once mounted on slides, frozen sections were kept at -20°C. Paraffin sections for immunocytochemistry were air dried at 37°C for at least 12 hours, whereas paraffin sections designated for other histological staining methods were dried at 58°C for 12 hours as a further precaution to prevent section detachment during staining procedures.

Before staining, all sections were deparaffinised by immersing them in two changes of xylene (5 minutes each) and rehydrated in a graded series of ethanols (100% and 70%) to water.

Staining and histochemical methods

The following stains and histochemical methods were applied to the skin sections:

- Haematoxylin and Eosin
- Sacpic
- Periodic-Acid Schiff's reagent
- Succinate Dehydrogenase histochemical technique

A detailed description of the staining protocols is given in the Appendix (page: A.1 to A.4).

Haematoxylin and Eosin (H & E)

Sections were stained with H & E for microscopic estimation of follicle density, fibre diameter and the ratio of secondary to primary follicles. This stain results in a blue-black colour of cell nuclei and other basophilic cytoplasmic structures. Other structures and tissue components stain in shades of pink to red. For estimation of secondary to primary follicle ratio and follicle density, sections cut at the mid-sebaceous gland level of primary follicles were chosen. The follicle identification criteria used is that of Hardy and Lyne (1956) where primary follicles are distinguished by the close association of an epitrichial/apocrine sweat gland duct, arrector pili muscle and sebaceous gland. In contrast, secondary follicles are accompanied only by sebaceous glands.

Sacpic (Auber, 1952, modified)

A special trichrome stain was used to demonstrate follicular changes in the inner root sheath during the annual cycle of wool growth. This stain 'sacpic' originally described by Auber (1952) is well suited for detailed demonstration of the tissue components of the skin as well as revealing different features of keratinization by differential staining. Serial transverse skin sections 8 µm thickness were stained with this method after deparaffinization and rehydration.

As a first step, a celestin blue-haemalum sequence of staining is applied which provides a powerful and precise nuclear stain, resistant to decolorisation by succeeding stains and solutions. Scott's tap water is used as a substitute for ordinary tap water to produce a rapid blueing reaction for haematoxylin. The second step uses the special keratin stain 'Winiwaters Safranin' which, after differentiation of the previously stained sections in alcoholic solution, leaves the keratinizing portions of the fibre cortex and keratinized parts of the inner root sheath deep red in colour. Finally, Picro-indigo-carmine stain provides a bluish-green colour to connective tissue. Differentiation after this last step should show the connective tissue bright blue as opposed to the paler green stain of the epithelium.

The stained skin sections appeared as follows:

- collagen tissue:	sharp blue
- smooth muscle:	bright green
- undifferentiated epithelium:	pale green
- trichohyalin droplets:	dark bluish green
- "keratinized" IRS:	red
- pre-keratinized cortex of fibre:	red
- keratinized cortex:	yellow
- keratohyalin:	green
- stratum corneum:	dark green
- nuclei:	crimson or purple

Figure M.1 is a photomicrograph of a cross-section of sheep skin cut at mid sebaceous gland level and stained with the sacpic stain.

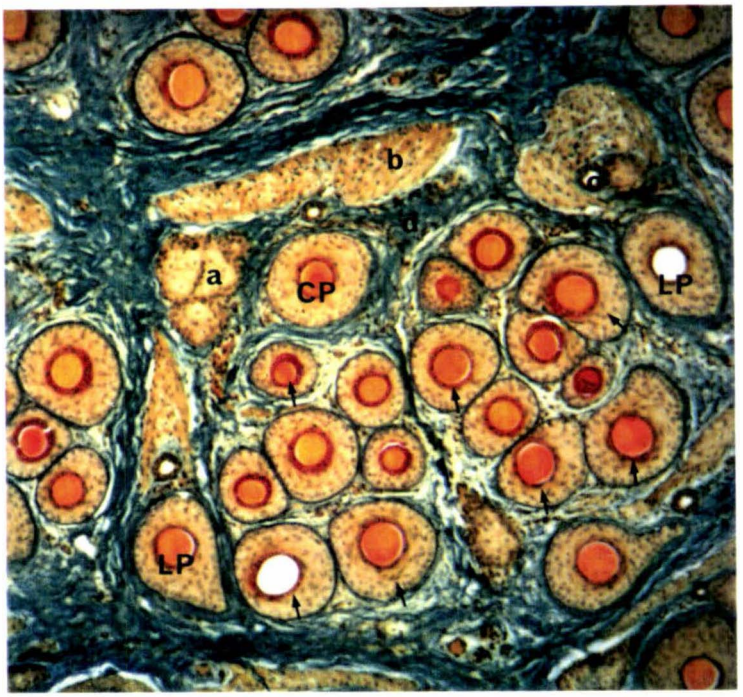

Figure M.1 **Cross section through a follicle trio group at mid sebaceous gland level stained with sacpic stain.** Magnification: 90x. **a:** sebaceous gland, **b:** smooth muscle, **c:** sweat gland duct, **d:** connective tissue, CP: central primary follicle, LP: lateral primary follicle, **arrows:** secondary follicles

Periodic-Acid Schiff reaction (PAS)

The principle of this technique, used to demonstrate carbohydrates, is based on the fact that certain tissue elements (hydroxyl groups of adjacent carbon atoms) are oxidized by periodic acid to aldehydes. These then react with Schiff's reagent to form a stable, pink or dark red reaction product (Culling, 1974). For the PAS procedure, cryostat sections were fixed with 10% formol saline prior to staining. If exact location of glycogen is required, this is believed to be a more suitable method since otherwise, in paraffin sections, the movement of fixative through the cytoplasm causes streaming of glycogen towards one side of the cell (Drury et al.,1967). In interpreting PAS reactivity, it must be borne in mind that there is a range of tissue compounds other than polysaccharides which show a positive reaction to PAS, namely:

- Glucosaminoglycans and Glucosaminoglucuronoglycans
- Glucoproteins and Glucopeptides
- Glycolipids
- unsaturated Lipids
- Phospholipids (Pearse, 1985)

In order to demonstrate cellular glycogen specifically, an enzymic control slide must be included. This consists of treating parallel control slides with diastase for 30 min at 37°C prior to staining. Incubation with diastase removes all glycogen from the tissue. Comparison between enzyme-treated and positive untreated control slides stained in parallel shows specifically the presence of glycogen.

This procedure shows PAS-positive substances pink-red, nuclei pale blue and cytoplasm yellow. Serial sections are scanned for presence or absence of glycogen in follicular structures and quantitative differences shown as different shades of red between treatment groups are noted.

Succinate Dehydrogenase (SDH)

The histochemical method originally described by Seligman and Rutenburg (1951) for the localization of SDH activity in fresh frozen sections demonstrates active SDH as granular depositions of a blue pigment intracellularly. In general, the demonstration of an enzyme is based on its specificity to a given substrate. Hence, in order to visualize an enzyme it has to be active. The enzyme itself cannot be seen, but the reaction it has with a substrate results in the formation of an insoluble, coloured complex at the site of enzymic activity (Culling, 1974). Due to the unstable nature of enzymes, it is essential to preserve them in a way that will achieve

maximum activity together with accurate localization. For this reason fresh frozen sections are used and negative and positive controls are performed in parallel. In case of SDH-demonstration, the negative control consists in omitting the substrate sodium succinate thus incubating sections in only phosphate buffer. The method for SDH is based on the fact that this oxidative enzyme reacts through a transfer of hydrogen from the substrate (sodium succinate) to a hydrogen acceptor (tetrazolium salt). The acceptor is, in turn, reduced from a colourless tetrazolium salt to a coloured formazan compound which forms the visible reaction product (Drury et al., 1967).

Microscopy and photomicrography

Slides were examined using a photomicroscope (Leitz Ortholux, Wetzlar, Germany) with a tungsten light source. Photomicrographs were taken on Agfachrome 50L (Agfa Gaevert N.Z. Ltd. Auckland) film using a camera (Leica 35 mm), with exposure times determined using a Microsix exposure meter. Colour prints in this thesis were reproduced from 35 mm transparencies.

Cell counting and statistical methods

Counting methods and statistical evaluation of data are described in the corresponding chapters of this thesis.

Chapter 1
Preliminary Investigations into the Follicle Morphology and Energy Metabolism of Fleeceweight and Control sheep

INTRODUCTION

Wool production differs considerably between breeds (Daly and Carter, 1955) and strains (Dunlop et al., 1966) of sheep under similar environmental conditions and feeding regimes. Factors such as hormones (Wallace, 1979, Williams et al., 1986 and Williams et al., 1990) and feed intake (Williams, 1979) have been found to influence wool growth.

At skin level, the follicles form the morphological basis of fibre formation and production. Variations in follicle morphology, resulting in functional changes of follicles, may be responsible for differences in wool production. These variations may be brought about by several genetic or environmental components. Ryder and Stephenson (1968) give a summary of principal ideas on components influencing wool growth and fleece structure. Response to selection for increased fleeceweight might be brought about by

- a change in dimensional and functional features of individual follicles,
- an increase in the number of follicles in the population (density),
- changes in fibre diameter or fibre length growth rate or
- changes in the metabolic efficiency of follicles to grow wool.

Follicle population parameters

Wool production can be assessed by measuring fleeceweight, which has proved to be highly heritable in sheep (Ryder, 1965). The components of clean fleeceweight were defined by Turner (1958) for medium-wool Merino sheep. Clean wool production per head was defined as the wool production per unit area of skin and the wool growing surface area. Wool production per unit area of skin, in turn, was determined by the mean fibre cross-sectional area, the mean fibre length and the mean number of fibres per unit area of skin. Work by Schinckel (1953), Carter and Clarke (1957a,b), Turner (1958) and Dun (1958) suggested that potential wool production might be directly related to S:P ratio i.e. it is the result of an increase in secondary follicles. Carter and Clarke drew direct relationships between the number of follicles in a group, the S:P ratio and wool production, but stated that these relationships only held within

breeds.

Fraser and Short (1958) proposed two explanations for an increase in wool production. First, that without influencing the amount of nutrients, wool production could increase either through increased efficiency of the follicles or through increase in the number of follicles with the same efficiency. Secondly, that a numerical increase of follicles occurred as response to selection for fleeceweight, while maintaining the fineness of the fleece. Dun (1958) and Turner et al. (1968) observed that selection response in density accounted for response in wool production per unit area of skin. The mean number of fibres per unit area has been considered as one of the most important contributors to wool production per unit area, when selecting for high wool weight with restricted fibre diameter (Turner et al., 1968). This is the case for Merino sheep. Schinckel (1957), Fraser and Short (1960) and Rendel and Nay (1978), suggested that the rate of wool production was not determined by follicle density. Dunlop (1962, 1963) stated that the variation between strains was associated with differences in diameter, length and density. Diameter and length appeared to be positively related, but both correlated negatively to density. On the other hand the overall difference in fibre diameter was small.

Most of these studies were conducted on Merino sheep, where fineness of fleece is a crucial parameter for the quality of the end product. Although the general principles about interactions between different parameters are valuable, application to the Romney sheep might need further investigation. Morphological changes in the skin and wool follicle associated with genetic differences in wool production in medium and strongwool breeds have rarely been investigated.

Henderson and Hayman (1960) working on New Zealand Romney lambs under different feeding regimes, studied the relationship between fleeceweight and its components, fibre density, fibre cross-sectional area and fibre length. Within groups of lambs, density and length were shown to be most closely related to wool production. Between groups, changes in wool production were mainly due to fibre length changes. In the New Zealand Romney, fibre diameter contributes to an increase in wool production (Blair, 1981). Fibre length, which was correlated to staple length, increased as a response to selection for greasy fleeceweight (Blair et al., 1985). Smooth body surface area was directly related to liveweight from which it is usually derived as a measure. Johnson (1981) and Blair et al. (1985) found correlated responses in liveweight to selection for fleeceweight.

Hence, it appears that depending on the breed and the related selection objectives or restrictions, responses to selection for fleeceweight can be brought about through changes in fleeceweight components. Consequently, for the current study, some of these components (S:P

ratio, density, fibre diameter, staple length, liveweight, woolweight and bodyweight) were investigated to elucidate their importance in the chosen experimental animals.

Energy metabolism

Glycogen

Hair follicle bulb cells are believed to divide faster than any other tissue in the mammalian body (Weinstein and Mooney, 1980). Cell proliferation as well as protein synthesis for fibre production and keratinization require a great amount of energy. Bullough and Laurence (1958) have shown in in vitro studies with mice that in hair follicle cells carbohydrates are necessary for cell division to occur. Thus, carbohydrates could also play an important role as a limiting factor in wool growth, their main function being to supply energy for metabolic processes via oxidation of glucose in the cell. Many tissues store glucose as insoluble glycogen which can be broken down (glycogenolysis) to glucose if required. Glycogen and other carbohydrates can be demonstrated in tissue sections histochemically with the Periodic Acid Schiff (PAS) reaction. Ryder (1958a) observed stored glycogen in wool follicles of sheep mainly in the cells of the ORS, with the greatest concentration in the lower half of the follicle above the bulb neck. There appeared to be variation between animals in the glycogen concentration but independence of concentration from the nutritional status of the animal. The bulb matrix cells did not show any glycogen. The greatest concentration in the fibre was found in an area starting at the bulb neck to the pre-keratinization region. The medulla frequently contained glycogen. The pattern of distribution of glycogen in follicle tissue described by Ryder concurs with the description of Montagna (1956) for human hair follicles and that of Hardy (1952) for murine follicles.

The actual function of glycogen in the follicle is still poorly understood. Ryder suggested that glycogen in the ORS served as an energy store and could be passed to the bulb as glucose via the capillary network. Glycogen in the fibre could supply energy for keratinization. The discovery of acid phosphatase in the nonkeratinized part of the fibre, possibly being associated with glycogen breakdown, supports this hypothesis.

There appears to be a relationship between the amount of glycogen in the follicle and the hair growth cycle. Ryder (1958b) observed that glycogen disappeared when the fibre moults, and specified that glycogen was removed during catagen rather than shortly before. Hardy (1952) and Montagna (1956) could not find any glycogen in quiescent follicles of rodents, but some glycogen was observed in the cells of the epithelial sac of resting human hair follicles. Shipman et al. (1955) found a relationship between the amount of glycogen in skin of mice and

the hair growth cycle but little or no glycogen during telogen and early anagen. Glycogen started to accumulate gradually as the active hair follicle reorganised. Shipman et al. (1955), Montagna (1956) and Braun-Falco (1958) suggested an inverse relationship of glycogen concentration at sites of normal keratinization and mitotically active areas. Glycogen in the ORS occurred mainly during a time when the ORS was relatively mitotically inactive. It was not identified in bulb matrix cells. Glycogen therefore might supply energy for protein synthesis necessary for keratinization. These observations pose the question of whether in fact there is any connection between glycogen storage and energy requirements for wool production in the follicle. It seems reasonable to conclude that energy stores for cell proliferation processes should be found much closer to the follicle bulb than in the cells of the ORS.

Nevertheless, wool production in genetically different sheep could be due to variations in energy metabolism of follicles in order to increase follicle efficiency, or show greater susceptibility to seasonal activity modulating forces, which could be expressed in different glycogen levels. Especially during the winter period, when production differences between the two lines are expected to be greatest, the amount of glycogen stored in the follicle might be subject to changes.

Succinate Dehydrogenase (SDH)

The tricarboxylic acid cycle is an aerobic oxidation process where carbohydrates, lipids and proteins are fully oxidized to CO_2 and water. All enzymes engaged in this cycle are found in the mitochondria matrix. SDH oxidizes succinate to fumarate and in turn reduces hydrogen transport flavoprotein (FAD > FADH). This then links into the electron transport chain of the cytochrome system. SDH has been investigated before in the epidermis with histochemical techniques (Ohkawara, 1979), its activity being topographically associated with regions of cell growth and energy consuming cell functions (Braun-Falco, 1958). Differential centrifugation and histochemical studies showed that much of the SDH activity is localised in and on the mitochondria (Formisona and Montagna, 1954). Tricarboxylic acid cycle enzymes are found in the matrix whereas the others are bound to the inner membranes of mitochondria. The SDH distribution appears to be similar in follicle cell mitochondria of mammals (Formisano and Montagna, 1954). In telogen, SDH exhibits less activity than in anagen where it is located in the follicular matrix bulb region (Rogers, 1953). No activity occurs in the keratogenous zone.

The main pathway for glucose utilization in the skin tissue and follicle seems to be glycolysis (Embden-Meyerhof cycle) where up to 95% of glucose is anaerobically oxidised to lactate (Philpott and Kealey, 1991), only up to 10% remains to be oxidized to CO_2 and water

through the tricarboxylic acid cycle. Nevertheless, especially since lipids and proteins feed into this cycle as well, it is considered to be an important energy source for the follicle (Ohkawara, 1979).

SDH could be used as an indicator for differences in metabolic rates of sheep genetically different in wool production. SDH located in the mitochondria of follicle bulb cells is positively related to aerobic activity of these cells. Differences in the amount of visible mitochondria in the follicle bulbs of FWT and CLT sheep may be an indication for genetic variations in metabolic activity connected to wool production.

Cell proliferation in the follicle bulb

The rate of wool fibre growth is largely determined by the proliferation of undifferentiated mitotically active cells in the follicle bulb and their subsequent hypertrophy, differentiation and sequential migration (Black, 1987). To gain a better understanding of the mechanisms controlling or influencing wool growth, cell kinetic parameters and their relationship have been investigated by the use of stathmokinetic agents like colchicine and colcemid which arrest multiplying cells in metaphase and thus allow counting of the number of arrested nuclei in a histological section (Schinckel, 1961; Fraser, 1963; Fraser, 1965; Wilson and Short, 1979). Autoradiography, based on the application of radioactive labelled DNA-precursors like ^{3}H-thymidine and examining either plucked fibre or histological skin sections, has also been used (Chapman, 1971; Chapman et al., 1980).

During the last decade, non-radiographic methods for distinguishing proliferating or DNA-synthesising cells have been developed. These use monoclonal antibodies 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; Dolbeare et al., 1983; Morstyn et al., 1983; Dean et al., 1984). Monoclonal antibody-antigen reactions are highly specific and easily applied to many tissues, both in vivo and in vitro. Their advantages over autoradiography and the use of metaphase arresting agents are their specificity and relatively short reaction time. Cell nuclei which have incorporated BrdU may be detected by various immunocytochemical methods and rendered visible for either brightfield or fluorescence microscopy (Gonchoroff et al., 1985; Gonchoroff et al., 1986; Morstyn et al., 1986; Czernielewski and Demarchez, 1987; DeFazio et al., 1987; Schutte et al., 1987; Penit, 1988). Recently, the in vivo application of BrdU through part or whole body infusion for cell proliferation studies in the follicle bulb of sheep was investigated (Hynd and Everett, 1990; Holle and Birtles, 1990) in combination with immunocytochemical detection methods for incorporated BrdU. A highly specific

immunocytochemical method to detect proliferating bulb cells in the wool follicles of New Zealand Romney sheep has been tested in the present study. The cells were labelled in vivo by infusion, using a surgical technique to isolate an area of skin on the lateral abdominal flank of the sheep (Harris et al., 1988, 1989).

This chapter investigates some characteristics of skin and follicle morphology as well as parameters believed to be essential for energy metabolism of skin and follicle tissue. Skin and follicle morphology of two lines of Romney sheep on genetically different levels of wool production was examined, to clarify the relative importance of certain morphological features to wool growth. Since the emphasis of this investigation was strictly concerned with morphological mechanisms, other influences were kept standardised as far as possible. The flock of sheep used in this experiment was described in detail in the general Introduction (page: I.26), which includes a review of studies already conducted. Selection for increased wool production in this flock was based on clean fleeceweight. At the onset of the project, neither the skin and follicle morphology, nor the follicle energy metabolism of these sheep, had been studied.

MATERIALS AND METHODS

Animal maintenance

In order to ensure that the animals were kept under standardized, controlled conditions, rams were brought indoors and housed in individual pens. The room was air conditioned, maintained at a temperature of 15-17°C and a constant artificial light with an intensity of approximately 500 Lux for 24 hours.

The animals were offered a diet consisting of 800-1000g/d lucerne chaff and water ad libitum. Daily food intake and body weights were recorded to provide an indication of the animals' health status throughout the experimental period.

Dye-banding

Details on the technique used to determine fibre length growth are given in the general Materials and Methods (page: M.2). Dye-banded fibre staples were measured in five different places on the animal by placing a ruler in direct contact perpendicular to the surface of the skin and holding the staple against it in order to determine the position of the dye-band. This was done without putting excessive tension on the wool. Data were recorded for the 5 different positions on the dye-banded staples and the mean fibre length per animal calculated. The time

interval between the application of dye to the wool and measurement of the dye-banded wool staples was 39 days during a trial period in winter. The data are given as daily growth rates. Fibre length growth rates were determined before the animals were brought indoors.

Skin histology

Details of the methods used for the collection of skin samples, both fresh and frozen, tissue preparation for histology and the histological staining principles and methods, including histochemistry, are given in the general Materials and Methods (page M.3) and the Appendix (page: A.1 to A.4)

Follicle population parameters

Follicle population density, S:P ratio and fibre diameters were measured on H&E stained cross sections of skin. For the determination of follicle population density and S:P ratios, counts were made on 10 fields of view per sample under the light microscope. An eyepiece graticule with a square grid in connection with a micrometer scale was used for the calibrations. All counts were made from sections cut at mid-sebaceous gland level, in order to identify secondary and primary follicles.

Fibre diameters were measured with a micrometer eyepiece as two internal diameters of the follicle (hair) canal, perpendicular to each other in order to account for oblique cuts through follicles. This diameter was chosen, since in the majority of cases, the fibre inside the hair canal was lost during tissue processing. Follicle density estimates were corrected for tissue shrinkage due to fixation and paraffin wax processing. Shrinkage factors for each sample were determined as described by Carter and Clarke (1957a).

Calculation of fibre volume

Fibre length and fibre diameter measurements were used to calculate fibre volume, assuming the fibre to be a cylinder.

Energy metabolism of individual follicles

Histochemical techniques described in the general Materials and Methods and in the Appendix were applied to determine the metabolic activity of individual follicles.

Glycogen

Longitudinal sections of wool follicles were examined for the occurrence of intracellular glycogen under the light microscope. The distribution of glycogen in various structures of the follicle was recorded. Variations in the amount of glycogen, as shown through different intensities in red colour of glycogen granules, were assessed using a visual colour grading system indicating:

[0] - no presence of glycogen

[1] - some traces of glycogen

[2] - apparent presence of glycogen

Diastase treated control slides were viewed concurrently to differentiate between colour reactions attributable to glycogen and reactions caused by other PAS-positive substances.

Succinate Dehydrogenase (SDH)

In longitudinal skin sections, 10 bulbs per animal were screened for the occurrence of dark colour precipitation over intracellular mitochondria. The number of reactive mitochondria was counted and the area of the corresponding bulb determined by tracing of the bulb outlines with a digitiser interfaced with a computer program (Sigma-Scan, 3.9, Jandel Scientific, Corte Madera, CA, USA).

Preparation of animals for immunocytochemistry of skin samples

Surgical techniques

General surgical preparation

Prior to surgery, the animals were given only 400 g lucerne chaff. Water was still available ad libitum. Anaesthesia was induced in all animals with Thiopentone Sodium BP (Pentothal 5g/100ml, Abbott Australasia Pty Ltd, Sydney, Australia). Anaesthesia was maintained with 2-3% (v/v) halothane (Fluothane, ICI, Macclesfield, Cheshire, UK) in oxygen delivered via an endotracheal tube at a rate of 2 l/min from a Fluotec 3 vaporiser (Cyprane, Keighley, UK). Surgical procedures were conducted under sterile conditions. After recovery from general anaesthesia, the animals were placed in sternal recumbency back into their individual pens. As a prophylactic antibiotic treatment an intramuscular injection of Terramycin (20% w/v, Pfizer) was given to each animal.

Surgical procedure

The sheep were catheterised in branches of the deep circumflex iliac artery and vein to allow infusion of substances into a defined isolated area of skin on the lateral abdominal flank.

The method for this arterio-venous preparation was developed by Harris et al. (1989) (Figure 1.1).

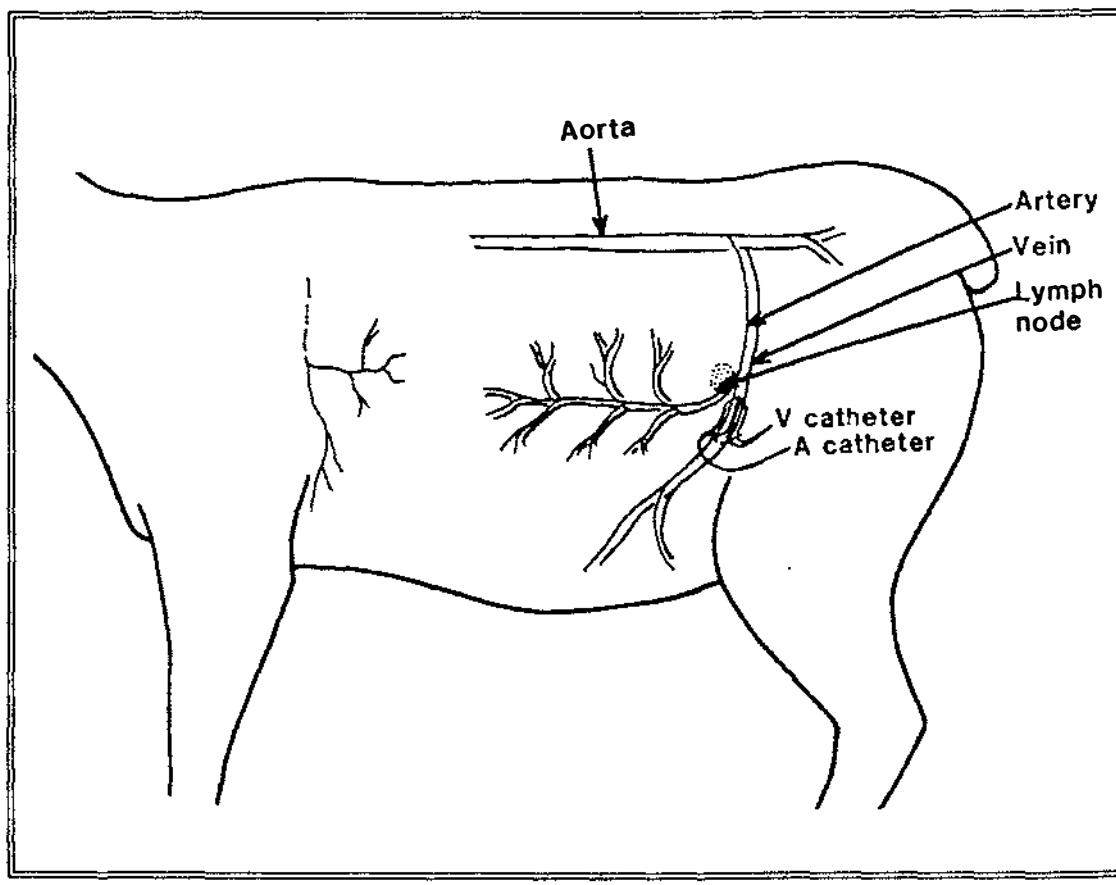

Figure 1.1 **Schematic presentation of the arterio-venous preparation of branches of the deep circumflex iliac artery and vein on the lateral abdominal flank of a sheep (Harris et al., 1989)**

Maintenance

Patency of the catheters throughout the experimental period was maintained by infusion (60 ml/day) with heparinised saline (30 IU/ml). For the infusion, the arterial catheters were connected to a peristaltic pump (Technicon). The flow rate was regulated by loading the pump with tubes of different internal diameters. Shortly before any experiment, the catheters were flushed with heparinised saline containing 100 IU heparin per ml. After recovery from general anaesthesia, the animals were kept in their crates for at least one week before they were used for any experimental work.

Infusion

The animals received an infusion of BrdU-solution (Sigma, 30 mg/kg) through the arterial catheter into the isolated skin area. The flow rate was defined by the internal diameter of the infusion line and held at 20 ml/hour. Infusion time was approximately 16 min and the exact period was recorded for each individual animal. Additionally, the amount of BrdU solution was measured by weight before and after the infusion to determine the mean infusion rate. Skin biopsies were taken from the infused area one and two hours after termination of the infusion. At the end of the experiment, the animals were euthanised with an overdose of barbiturate (Sagatal, May and Baker, Dagenham, U.K.). The isolated skin area was defined in each animal by injection and tracing of coloured latex to ensure that the biopsies were taken at the right sites. The area was measured, followed by dissection and separate weighing of the skin and the muscle layer. Additional data of body weight, total skin weight and greasy wool weight were recorded.

Immunocytochemical localisation of incorporated BrdU in skin sections

Two techniques were evaluated for the immunocytochemical detection of incorporated BrdU. The indirect immunoenzyme technique, described first, produced excessive non-specific background staining and was therefore replaced by the biotin-streptavidin technique.

Indirect immunoenzyme technique

Tissue sections were deparaffinised in two changes of xylene and rehydrated in a graded series of ethanol washes to water. After rinsing in 0.01 M phosphate buffered saline (PBS), pH 7.4, the slides were treated in 1% bovine serum albumin (BSA) in PBS for 5 min to block non-specific tissue binding sites. The slides were then incubated in mouse anti-BrdU antibody

(Amersham International) for 60 min at room temperature in a humidified chamber. Since this formulation of monoclonal antibody contains the enzyme nuclease, it was not necessary to denature cellular DNA as a pre-incubation step to allow antibody access. To ensure the specificity of the monoclonal antibody reaction, several controls were applied. Skin sections from the same tissue samples were covered with an inappropriate antibody (bovine anti viral diarrhoea antibody) diluted 1:1000 in 1% BSA in PBS or remained covered with the 1% BSA while omitting the mouse anti-BrdU antibody. Additionally, tissue sections of untreated skin samples were covered with mouse anti-BrdU. After incubation with the primary antibody, all slides were washed three times in PBS, for 1 min each time, and incubated for 30 min under the conditions described above with peroxidase-conjugated anti-mouse IgG (Amersham International). Following another three washes in PBS, the slides were incubated for 5 min in 0.005% diaminobenzidine solution (DAB) made up from a 1 ml aliquot of DAB solution (Amersham International) in 50 ml of PBS plus 5 drops of a cobalt-nickel formulation. The enzyme peroxidase polymerises DAB in the presence of cobalt and nickel and thus stains black-brown at sites of BrdU incorporation in the tissue section. The slides were washed in distilled water, counterstained with 0.1% acetic light green for a few seconds, dehydrated through graded ethanol, cleared in xylene and mounted in DPX. Cells that had incorporated BrdU were identified by the black-brown colour of their nuclei.

Biotin-streptavidin technique

The detection portion of the immunocytochemical technique described above was changed in favour of a biotin-streptavidin detection system. Instead of peroxidase-conjugated anti-mouse IgG, the second step consisted of incubation with biotinylated anti-mouse IgG (Amersham International) diluted 1:200 in 1 % BSA in PBS for 30 min under the conditions described above. As a third step, streptavidin-biotinylated horseradish peroxidase complex (Amersham International), diluted 1:300 in PBS was applied for 15 min at room temperature. Peroxidase was detected by incubating the slides in a DAB solution with heavy metal intensification for 3-4 minutes at room temperature. The solution was prepared by adding 4 mg of 3, 3'-diaminobenzidine (Sigma), 10 µl of 30% w/v hydrogen peroxide and 10 µl of a 1% solution of nickel chloride and cobalt chloride to 8 ml of 0.01 M PBS, pH 7.4.
This method virtually eliminated non-specific background staining while maintaining strong specific immunoreactivity in nuclear sites (Figure 1.2, A and B).

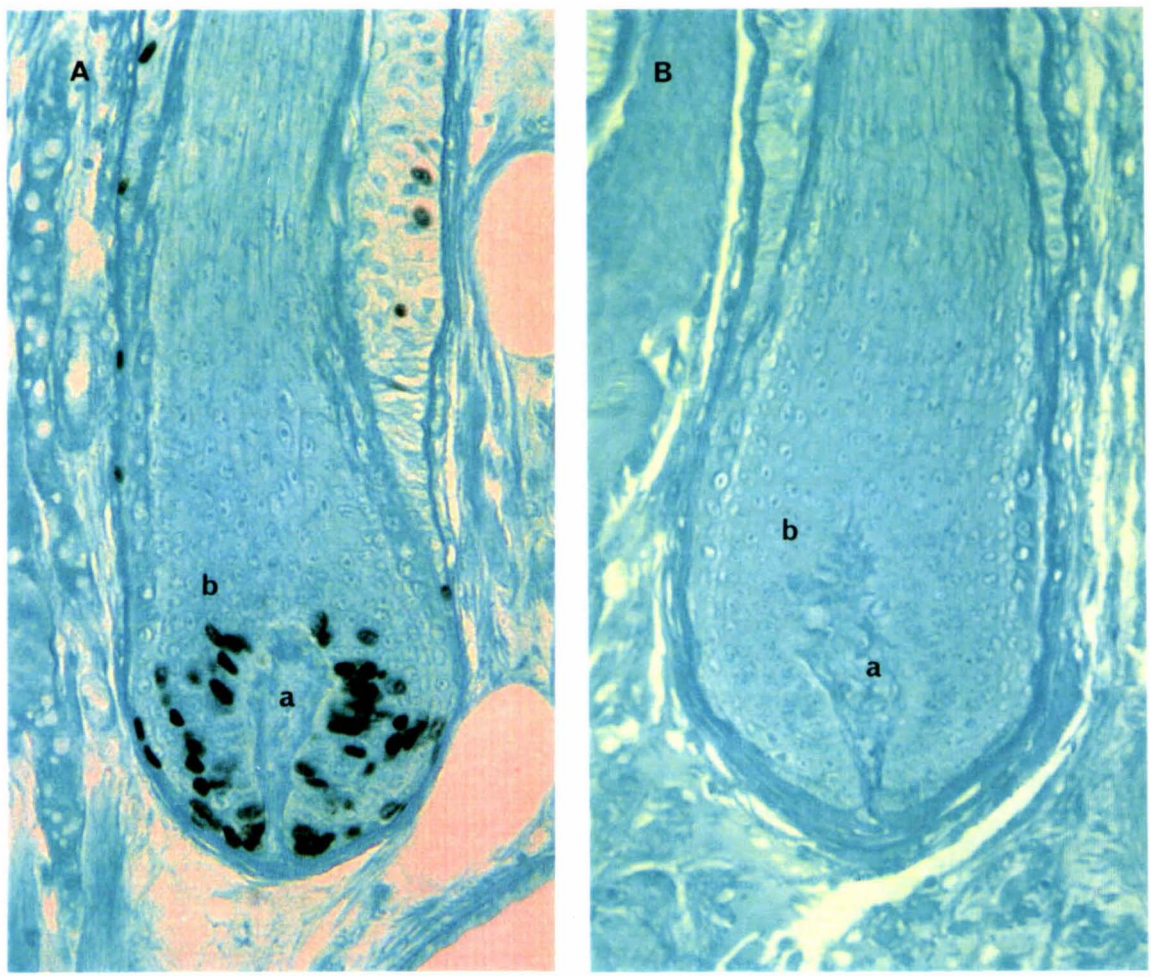

Figure 1.2 **Longitudinal sections through the lower parts of a wool follicle. Counterstain 0.1 % acetic light green. Magnification: 288x. a: dermal papilla; b: follicle bulb**
(A) immunostained with the biotin-streptavidin method for BrdU incorporation into cellular DNA.
(B) example of a negative control (omission of anti-BrdU antibody) to the section in Figure 1.2 (A), showing absence of immunoreactivity.

RESULTS

Fibre length growth rate and fibre diameter

Table 1.1 **Difference in daily fibre length growth rates (FL) (mm d^{-1}), fibre diameter (FD) (µm) and fibre volume (mm^3 d^{-1}) for CLT and FWT sheep tested by analysis of variance**

	FIBRE LENGTH			FIBRE DIAMETER			FIBRE VOLUME		
	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM
CLT	11	0.35	0.11	9	83.4	0.35	8	$2.0X10^{-3}$	$0.4X10^{-3}$
FWT	10	0.41	0.02	8	92.1	0.44	7	$2.8X10^{-3}$	$0.2X10^{-3}$
F	2.99			2.43			3.30		
p	NS/marg.			NS			0.09/NS		

Length growth rates and fibre diameter differences did not reach significance but showed a trend toward FWT sheep having an advantage (Table 1.1). If length growth rates and fibre diameters were used to calculate the volume of fibre output, FWT sheep showed a trend toward a greater fibre volume.

Follicle population density and S:P ratio

Table 1.2 **Differences in follicle density (mm^2) and S:P ratio of CLT and FWT sheep**

	DENSITY			S:P RATIO		
	MEAN	SEM	N	MEAN	SEM	N
CLT	29.4	1.75	12	6.10	0.13	10
FWT	27.5	2.01	9	6.04	0.16	9
F	0.47			0.07		
p	NS			NS		

Differences in S:P ratio and density were small and not significant (Table 1.2).

Energy metabolism

Table 1.3 **Mean mitochondria counts per bulb section, as detected by SDH activity in longitudinal sections of wool follicle bulbs for individual sheep**

GROUP	IDENTIFICATION NUMBER	MEAN	SEM
CLT	15	98.4	23.7
	113	97.8	11.2
	219	62.2	6.0
	144	70.7	18.6
	11	62.7	9.05
	131	91.6	10.6
	259	143.5	16.3
	184	81.8	15.2
FWT	104	93	14.1
	251	76.7	12.6
	258	193.4	22.3
	231	36.6	3.75
	105	47.3	9.52
	208	95.9	17.4

Table 1.4 **Difference in mitochondria counts, as detected by SDH activity, between CLT and FWT sheep, tested by analysis of variance**

GROUP	MEAN	SEM	N	F	p<0.05
CLT	88.59	5.73	80	0.04	NS
FWT	90.48	8.72	60		

Table 1.5 **Differences in follicle bulb area (μm^2) of CLT and FWT sheep, tested by analysis of variance**

GROUP	MEAN	SEM	N	F	p<0.05
CLT	115.62	4.63	80	0.05	NS
FWT	114.15	4.78	60		

Table 1.6 **Differences in mean index values (SDH/area) (counts/μm²) of SDH density between CTL and FWT sheep, tested by analysis of variance**

GROUP	MEAN	SEM	N	F	p<0.05
CLT	0.8683	0.0683	80	0.8	NS
FWT	0.7841	0.4621	60		

Table 1.7 **Colour gradings for PAS-positive carbohydrates and their occurrence in the basement membrane (BM) or outer root sheath (ORS) in the follicle for individual animals in CLT and FWT sheep** (*) reaction only in some follicles

CLT sheep

SHEEP NUMBER	COLOUR GRADING (no diastase)	LOCATION	COLOUR GRADING FOR diastase treated CONTROL
15	1	BM	1
	1	ORS	1
113	0	BM	0
	0	ORS	0
144	1-2	BM	1-2
	0-1	ORS	0
11	2	BM	1
	0-1	ORS	0
131	1	BM	1
	1-2	ORS	1-2
259	1	BM	0
	1*	ORS	0
184	2	BM	1
	1-2*	ORS	0
126	0	BM	0
	0	ORS	0
155	1	BM	0
	1-2*	ORS	0

FWT sheep

SHEEP NUMBER	COLOUR GRADING (no diastase)	LOCATION	COLOUR GRADING FOR diastase treated CONTROL
104	2	BM	1
	2	ORS	0
251	0	BM	0
	0	ORS	0
258	0	BM	0
	0	ORS	0
231	0	BM	0
	0	ORS	0
105	1	BM	0-1*
	0-1*	ORS	0
208	0-1*	BM	0
	0-1*	ORS	0
233	1	BM	1
	0	ORS	0

The mean values for mitochondria counts showed a wide within animal as well as within group variation with large SEM (Table 1.3). The group means of mitochondria counts were similar between lines of sheep, with no statistical significance in the difference of SDH reactive mitochondria (Table 1.4). Table 1.5 shows the group means of longitudinal bulb areas which again did not exhibit a significant difference between the groups. When the SDH/mitochondria counts were corrected for bulb area (SDH/area) to express the SDH density index (Table 1.6) again no significant difference was found between treatment groups.

PAS-positive substances were observed mainly throughout the entire length of both the ORS and the basement membrane of the follicle (Table 1.7). Some traces were occasionally observed in the dermal papilla. No other follicular structure demonstrated PAS positive reactivity. In the diastase treated control slides, the colour reaction was frequently pink-red rather then orange-red. If the ORS showed no reaction in the diastase treated control, the colour in the basement membrane appeared brighter than that in the untreated slide. When reactivity was found in the ORS, it was located along the entire length of the follicle and as in the case

of sheep No. 155 (Table 1.7A), even more was seen in the portion closer to the skin surface. Some reactivity, especially in sheep No. 184 (Table 1.7A) was located in the dermal papilla. In cases where PAS reactivity was observed in similar degree in both untreated and negative control slides, as in sheep Nos. 15, 131 and 233, this reactivity was attributed to PAS-positive substances other than glycogen. From the total of 16 sheep, 8 (50%) did not react for glycogen in the ORS. They did however show varying amounts of other carbohydrates. From the 8 sheep showing reaction for glycogen, 4 belonged to the FWT and 4 to the CLT. One (No 144) from the CLT and one (No 104) from the FWT reached the highest of the observed gradings. The remaining six had only traces of glycogen, but these were consistently seen in the ORS. There was however a wide variation between follicles. No pattern in the distribution differences between groups could be observed, whereas variations between animals within a group was rather pronounced. No glycogen was observed in fibres except in one follicle in sheep No. 184.

Whole sheep data

Table 1.8 **Bodyweight (kg), skinweight (kg) and woolweight (kg) measurements of CLT and FWT sheep**

GROUP	BODYWEIGHT		SKINWEIGHT		WOOLWEIGHT	
	MEAN	SEM	MEAN	SEM	MEAN	SEM
CLT	55.89	1.96	3.956	0.152	2.274	0.277
FWT	52.24	4.73	4.386	0.296	2.679	0.352

Table 1.9 **Analysis of variance F-values and significant levels for the three parameters in Table 1.8**

PARAMETER	N CLT/FWT	F	p<0.05
Bodyweight	9/5	0.69	NS
Skinweight	8/5	2.06	NS
Woolweight	8/5	0.82	NS

Table 1.10 **Ratio of skinweight to bodyweight (S/W) and woolweight to bodyweight (W/W), significance levels for analysis of variance for the group difference and percentage advantage (%) of FWT over CLT sheep**

	S/W		W/W	
GROUP	MEAN	SEM	MEAN	SEM
FWT	0.085	0.005	0.051	0.005
CLT	0.071	0.001	0.041	0.004
F	10.21		2.38	
p	0.009		0.151	
% FWT-CLT/CLT	18.3		24	

Table 1.8 shows group means and SEM of FWT and CLT sheep for bodyweights, skinweights and woolweights collected at the end of the experiment. There were no significant differences in either bodyweight, skinweight or woolweight between the groups (Table 1.9) at the 0.05 significant level. However, FWT sheep were generally lighter than CLT sheep but had heavier skinweights and relatively heavier woolweights. Taking the ratios of woolweight to bodyweight and skinweight to bodyweight, in the skinweight to bodyweight ratio FWT sheep had a 18.3% advantage, and in the woolweight to bodyweight ratio they had a 24% advantage over CLT sheep (Table 1.10).

Cell proliferation in the follicle bulb

Table 1.11 **Number of proliferating BrdU-labelled follicle bulb cells 1 hour after infusion, and analysis of variance for the group difference**

GROUP	MEAN	SEM	N	F	p<0.05
CLT	34.89	4.94	8	0.23	NS
FWT	39.20	5.78	3		

Table 1.12 **Number of proliferating BrdU-labelled follicle bulb cells 2 hours after infusion, and analysis of variance for the group difference**

GROUP	MEAN	SEM	N	F	p<0.05
CLT	49.20	8.56	8	0.00	NS
FWT	49.79	7.94	4		

Table 1.13 **Differences in the number of labelled cells per follicle bulb section from 1 to 2 hours after infusion, tested by analysis of variance**

GROUP	N	F	p<0.05
CLT	16	2.10	NS
FWT	7	1.00	NS

The number of proliferating cells counted in longitudinal bulb sections both 1 hour (Table 1.11) and 2 hours (Table 1.12) after infusion did not show significant differences between groups. An increase in the number of labelled cells within groups with time after infusion was apparent from differences between the 1 and 2 hour samples (Table 1.13) but was not significant.

DISCUSSION

This chapter has presented preliminary work undertaken to establish follicle variables of interest, in the study of genetic differences in wool production in Romney sheep. Several new procedures were developed and applied concurrently. This developmental aspect of the project resulted in the loss of samples and data in some instances.

Fibre growth

For the assessment of fibre growth rates, the animal numbers in each group appeared to be sufficient, yet the method of measuring the length of the dye-banded wool staples by using a ruler was unsatisfactory. The method can introduce several errors due to handling and possible stretching of the staples and consequently measurement difficulties. Although the principle of applying dye to wool as a marker for growth is widely used, since its introduction by Chapman and Wheeler (1963), it seems to be necessary to introduce a less error prone method to measure fibre length between bands, by assessing individual fibres rather than whole staples. Errors introduced through the method of measuring fibre length may well exceed existing fibre length differences between lines. A more precise, method was therefore developed to measure

individual fibres, and is reported in Chapter 4.

Since the results did not show significant differences in either S:P ratio or follicle density, it does not appear likely that selection for fleeceweight has affected these parameters.

It is therefore unlikely that an increase in animal numbers would affect the results achieved in this study, but more likely that improved methodology in some measurements would improve the ability to detect small differences between the lines in some measured variables. However, the measurements in the present chapter were only conducted during winter. Other periods of the year may well show different results. This could be especially the case in a breed showing a seasonal pattern in wool production (Chapter 4).

Fibre diameter measurements, measured within the follicle canal, although not significant, did show a trend towards FWT animals having greater diameters than CLT. Further measurements, on fibres external to the skin as well as intrafollicular, may well reveal a significant difference. Differences in fibre volume output were apparent but more animals might be needed to establish a significance in the superiority of FWT sheep.

Glycogen in the basement membrane

Montagna (1956) observed that the inner layer of the basement membrane and the dermal papilla were PAS positive but diastase resistant, which is in agreement with findings in this study. The positive reaction is therefore due to carbohydrates other than glycogen. Since glycogen is stored intracellularly and the basement membrane is an extracellular structure, there is no possibility of storing glycogen. The reactivity in the dermal papilla might be attributed to other PAS positive substances in portions of the basement membrane enclosing the papilla and in the basement membrane of capillaries.

Glycogen in the outer root sheath

The wide variation between follicles accords with the findings of Ryder (1958a). There was a surprisingly low amount of glycogen in all follicles, in spite of their being in anagen (Introduction, page: I.24). According to Hardy (1952), Montagna (1956) and Ryder (1958b, 1965), the anagen follicle should show considerable glycogen in the ORS, an observation not supported by the current investigation. Glycogen stores are believed to disappear during catagen, not before, so that the absence in an anagen follicle here does not seem to indicate an association with cyclic hair growth events.

The significance of glycogen in the wool follicle

A hypothesis was put forward (Ryder and Stephenson, 1968) that glycogen in the ORS could be used by the follicle in times of nutrient shortage in the blood. Because wool and hair grow continuously throughout 24 hours, the follicle actually has to compete for glucose with other body tissues, regardless of diurnal changes in nutrient supply. Investigations with mice have shown (Ryder, 1958b) that once a follicle is mature and has its glycogen stores, poor diet does not prevent hair growth but can only slow it down, whereas poor diet at the start of a cycle, i.e. before the establishment of glycogen stores, can prevent hair growth. In contrast, Loewenthal and Montagna (1955) observed that there was glycogen in the follicles of mice as long as the hair was growing, no matter what the nutritional state of the animal. A possible explanation for the present result is that the experiment was conducted during the winter, and food shortage could have affected the follicular glycogen stores. Wool production declines during winter (Story and Ross, 1960; Sumner and Wickham, 1969; Bigham et al., 1978), this decline eventually being partly caused by energy, i.e. glucose, shortage due to poor diet over the winter months. If this is the case, it is surprising that indoor housing of the sheep for 2-3 weeks with daily plentiful diet had no effect on reestablishing those glycogen stores. No glycogen was found in the fibres themselves. Ryder (1958a) suggested that the glycogen in the prekeratogenous zone is stored in order for the fibre to draw energy for direct fibre producing processes during keratinization. Again, the absence of glycogen could be explained by the seasonal effect of winter. From these findings it is highly questionable whether glycogen stores have any association with wool growth at all. If the ORS is the local storage site, glycogen would need to be broken down and then transported via the blood stream through the whole body first, before returning to the follicle bulb, via the capillary network of the dermal papilla, where it is needed as energy source. It appears unlikely that there is a more direct way (such as a portal system for each individual follicle) to transfer glycogen in the ORS to the bulb cells where energy is needed. Hence, glycogen in the ORS might well be utilised only for the energy requirement of the ORS cells themselves.

Significance of Succinate Dehydrogenase (SDH)

Differences between FWT and CLT sheep in the amount of SDH, as measured through counts of detectable mitochondria in the follicle bulb were not apparent. Furthermore, the number of mitochondria counted was very low suggesting that they, or oxidative phosphorylation, do not play a major role in energy metabolism of the wool follicle. In fact, this observation is in accord with studies on isolated hair follicles showing that as little as 10% of

available blood glucose is oxidised to CO_2 aerobically (Philipott and Kealey, 1991).

Whole sheep data

The results on woolweight and skinweight to bodyweight support the superiority of FWT sheep over CLT in terms of wool production. Especially, the ratio woolweight to bodyweight accounted for almost the entire difference reported for these sheep by McClelland et al. (1987b).

Experimental problems

Problems associated with the postsurgical period were mainly due to infections, particularly of the leg region, non-patency of the catheters, or shifting of the catheter tips with corresponding changes of the skin area supplied by these catheters. If catheters could not be kept patent, the animal was removed from the experiment, as also were occasional sheep which developed postoperative infections. These problems decreased animal numbers in both groups during this trial period to levels which did not yield statistically justifiable results for some of the measured parameters.

Difficulties which had to be overcome in order to develop the BrdU infusion technique produced very little quantitative data at the end of the experiment. The concept was however sufficiently encouraging, so that in order to elucidate the biological basis for genetic variation in wool production, further studies toward the cellular events in the follicle and changes in follicular dimensions were undertaken. Therefore, as a next step, a method for local intracutaneous BrdU administration had to be developed to avoid surgical modification of the animals (Chapter 2).

Chapter 2
Studies on the Kinetics of in vivo Labelling of Proliferating Follicle Bulb Cells with 5-Bromo-2′-deoxyuridine (BrdU)

INTRODUCTION

Wool production is determined by the total mass of the germinative tissue, the number and proliferation rate of bulb matrix cells, their subsequent migration and the proportion of these cells ultimately forming the fibre. Suitable methods to study and quantify the rate of cell division in the germinative region of the follicle bulb have long been sought to investigate one of the major factors influencing wool production. Previous investigations have involved stathmokinetic drugs (Schinckel, 1961; Fraser, 1963; Wilson and Short, 1979; Hynd et al., 1986; Hynd, 1989a) or radioisotopes (Ryder, 1956; Downes et al., 1966; Chapman et al., 1980). For in vivo wool growth studies, these agents were administered intravenously (i.v.) as well as intracutaneously (i.c.). Advantages and disadvantages of the abovementioned methods have been reported and discussed by Bertalanffy (1964), Tannock (1965) and Hynd et al. (1986), and demonstrate that none of these methods is suitable to account for all problems in cell kinetic studies. The ideal labelling agent would have: a) convenient administration of the label, b) safety for the operator and non-toxicity for the animal, c) simplicity in collection and processing of samples, d) a fast and convenient detection method for proliferating cells and e) known free and bound kinetics of the label to promote accurate interpretation of the 'true' cytokinetic and cytodynamic situation in vivo.

Time course labelling

The simplest model of cell dynamics in epithelial tissues is based upon linear kinetics. A linear rate of increase of blocked metaphase cells up to 5-6 hours after administration of stathmokinetic agents was investigated both for i.v.injections (Schinckel, 1961; Wilson and Short, 1979) and i.c. application (Hynd et al., 1986). For ^{3}H-thymidine some information is available on subcutaneous injections in new born pigs (Smith and Jarvis, 1978), reporting a linear increase of labelled cells in the intestine up to 96 hours. Chapman et al. (1980) administered ^{3}H-thymidine i.v. to Merino sheep and noted the position of labelled cells in the wool follicle as well as their migration through various zones of the follicle.

Label pharmacokinetics

Although information is available on the amount of ^{3}H-thymidine necessary to detect the label autoradiographically, few studies have been conducted to answer the question of free label availability resulting from its metabolism and clearance. The free pool of label around cell populations needs to be sufficient for all proliferating cells to incorporate that label and to be subsequently detected histologically. Again, no studies have shown the threshold free pool concentration which permits histological detection. Depending on the availability of the free label, any observed increase in the number of labelled cells with time may be a reflection of proliferating cells incorporating label and entering the labelled population, or, with longer time course experiments, previously labelled cells undergoing further division, or a combination of these effects.

Interpretation of time course labelling of cells is therefore dependent on the knowledge of changes in the concentration of free label available to the proliferating cell at any time, which in turn is dependent on the method of administration. Continuous infusion by i.v. administration will result in an increasing concentration of label which may eventually reach a steady state plateau when the infusion rate becomes matched by whole body clearance. A pulse of label administered i.v. will result in a rapid rise of label concentration in the plasma, intercellular and intracellular compartments, as it is distributed around the body and transported across cell membranes. The rate of decline of concentration will relate to whole body clearance. An i.c. injection could behave as a local pulse of label with the rate of decline in the available pool related to lymphatic and plasma clearance of the label from interstitial and intracellular fluid.

The use of bromodeoxyuridine (BrdU) as a labelling agent of proliferating cells in the bulb of the wool follicle and their immunocytochemical detection utilizing a monoclonal antibody has been previously reported (Holle and Birtles, 1990; Hynd and Everett, 1990). Bromodeoxuyridine (BrdU), a thymidine analogue, is incorporated into nuclear DNA during cell cycle S-phase and can be visualized with immunocytochemical techniques using a monoclonal antibody (Gratzner, 1982; Dean et al., 1984). In both studies, BrdU was administered systemically through infusion. Few data are available on the pharmacokinetics of BrdU. Greenberg et al. (1988) measured venous plasma BrdU levels with HPLC in the treatment of malignant astrocytomas in humans. They assumed linear kinetics of the drug distribution and metabolism with negligible clearance by the lungs. The systemic venous blood levels of BrdU after intraarterial infusion measured 0.18 μM with a total body clearance ranging from 0.99 to 11.3 litres/unit at an infused dose of 400-600 mg/m^{2}/day. DeFazio et al. (1987) measured plasma levels of BrdU after a single intraperitoneal injection of 200 mg/kg. From a

concentration of 35.2 µM after 30 min, the plasma levels constantly decreased to 9.3 µM after 6 hours. Simultaneously measured thymidine levels were only 2.5 µM. DeFazio concluded that higher levels of BrdU might aid in competing against endogenous thymidine pools for incorporation into DNA. These results emphasise the necessity of determining dilution rates of the labelling agents at the location of the sample site in order to draw precise conclusions on cell population kinetics.

Tocixity

In contrast to ^{3}H-thymidine, BrdU is neither radioactive nor myelosuppressive at the doses used for labelling studies in vivo (Hoshino et al., 1985). Marginally high dose rates and long exposure times have been used in humans to radiosensitize patients for subsequent cancer treatments (Greenberg et al., 1988) toxic side effects being myelosuppression and photosensitivity (Morstyn, 1983). In cell cultures, high dose rates and prolonged exposures was reported to cause cytocidal and teratogenic effects (Szybalski, 1974; Goz, 1978). The original development of BrdU was as an active growth inhibitor of bacteria. Littlefield and Gould (1960) suggested, from studies on cultured epithelial cells, that BrdU does not interfere with the first step of DNA replication. Cells exposed to BrdU contained a normal amount of DNA even after the majority of DNA-thymidine was replaced. They considered that BrdU-labelled DNA can still participate in mitosis but BrdU in the DNA would interfere with the DNA function such as the transfer of information required for protein synthesis.

This suggestion may relate to the recent observations of Adelson et al. (1991) on the occurrence of abnormal cuticle scale patterns and disturbance in the expression of ortho-and paracortex specific proteins in wool fibres of sheep previously injected with a BrdU/Fluoro-deoxyuridine (FdU) mixture (i.v.; 450 mg BrdU and 50 mg FdU/10ml). Despite visible disturbance at the fibre level, cell migration rates at the bulb level were similar to those recorded using ^{3}H-thymidine or colchicine as a label. Thus, cell proliferation rates appear to be unaffected by BrdU, but especially for the higher doses used by Adelson et al. (1991), it seems possible that cells with BrdU-containing DNA may lose the ability to participate normally in protein synthesis during subsequent cell differentiation and maturation.

In conclusion, it is apparent that a method should be developed using low BrdU dose rates producing minimal cytotoxicity. This includes the acquisition of a better method of application which excludes the hazards associated with whole body infusion techniques. This chapter tries to validate the use of an intracutaneous injection technique of BrdU for field studies, and describe the time course of numbers of labelled cells in the wool follicle bulb in

relation to the available free pool of BrdU.

MATERIALS AND METHODS

Sheep treatments

The sheep used in all experiments were rising two-tooth New Zealand Romneys. BrdU was prepared as a solution of sterile physiological saline and 0.1 ml injected intracutaneously with a tuberculin syringe (McIntock; Ewos Ltd., Bathgate, West Lothian, Scotland) to a depth of 4 mm, perpendicular to the unstretched clipped skin surface. Skin samples were taken with a 10 mm diameter trephine. Due to the large number of samples taken from each sheep, the animals were euthanised with an overdose of barbiturate (Sagatal; May and Baker, Dagenham, U.K.) immediately prior to sampling.

Experiment 1: Validation of intradermal injection of BrdU and accumulation of labelled S-phase nuclei with time

BrdU solution (3 mg/ml, sterile saline 0.9 % w/v) 0.1 ml was injected intracutaneously into several sites on clipped midside patches of six sheep. Injections were administered in sequence at 5, 15, 30, 45 min and 1, 2, 4, 6, 8, 10, and 24 hours before euthanasia and sampling. The distance between any two sites was a minimum of 30 mm (Figure 2.1,a).

Experiment 2: Localisation of BrdU labelling effect

The aim of this experiment was to determine the diffusion radius of BrdU into the surrounding tissue from the point of injection, as seen by the occurrence of labelled cells. A site on a clipped midside patch was injected with BrdU solution as described above and marked with a felt pen. After 2 hours the sheep was euthanised and skin samples were taken from the injection sites as a positive control for BrdU uptake into nuclear DNA. Additionally, samples in four directions (cranial, caudal, dorsal, ventral) from the injection site were taken at distances of 5, 25 and 50 mm (3 sheep) and of 50, 100 and 200 mm (3 sheep) from the edge of the sample over the injection site (Figure 2.1,b).

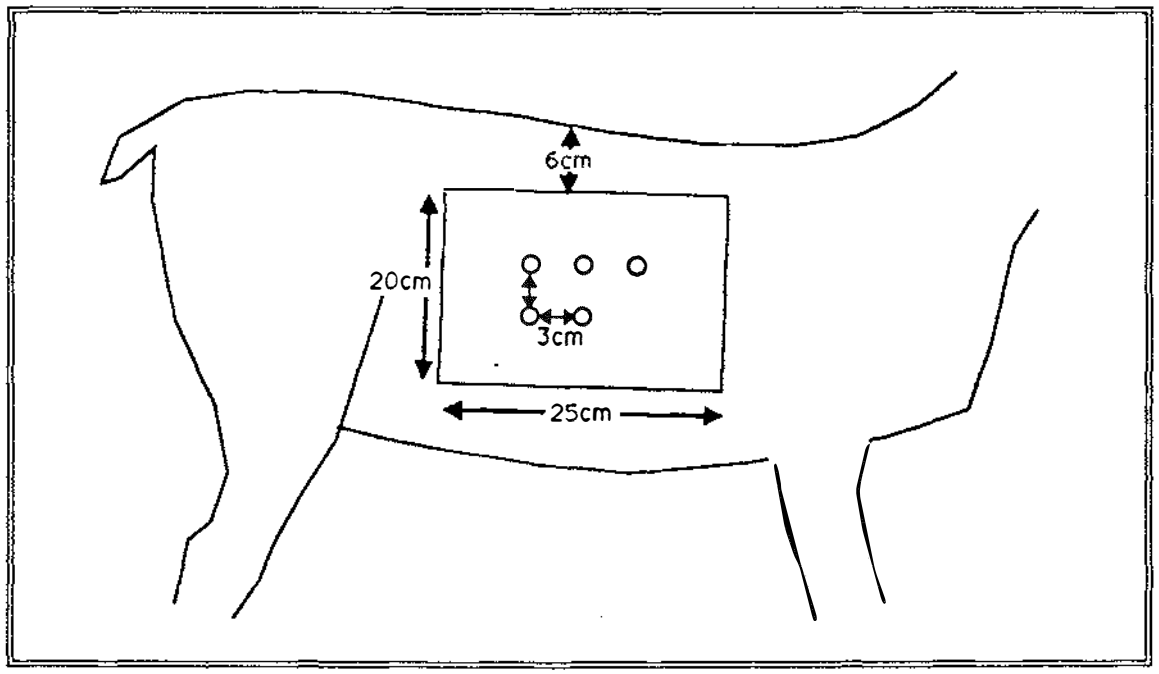

Figure 2.1,a. **Sampling sites on the clipped midside patch area for Experiment 1**

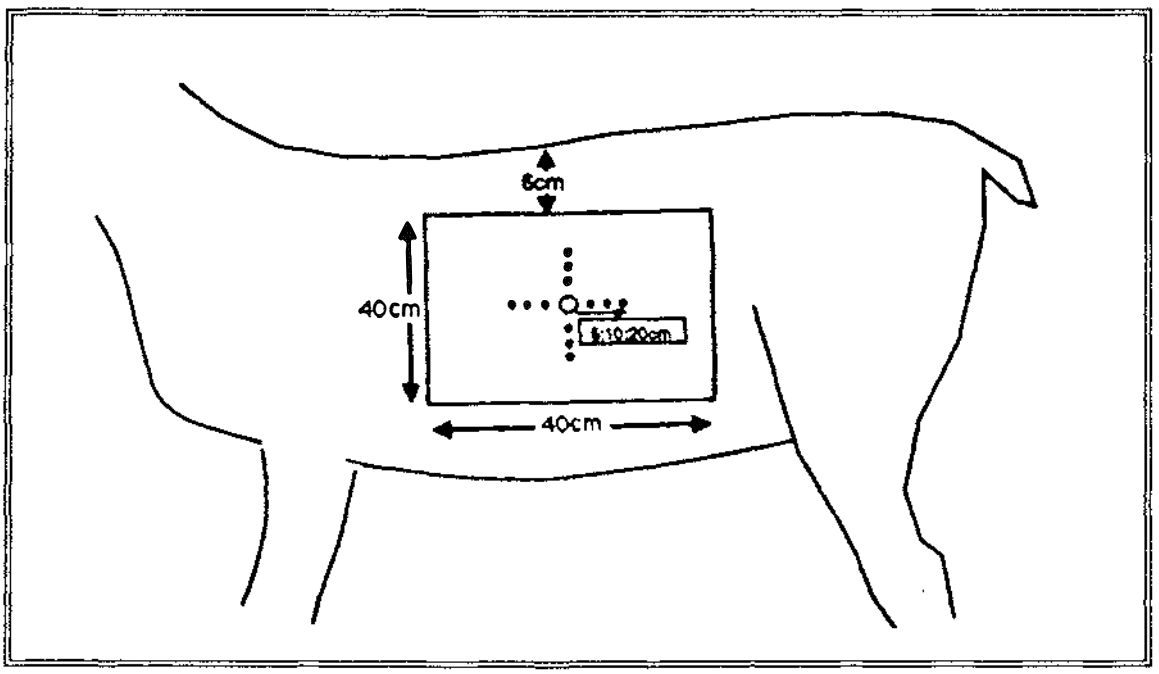

Figure 2.1,b. **Sampling sites and BrdU inoculation site on the midside patch area for Experiment 2. (•) indicates sampling sites, (○) indicates BrdU inoculation and sampling site**

Experiment 3: Changes in the concentration of free BrdU in skin tissue with time after injection (BrdU clearance rate)

One sheep was repeatedly injected i.c. at its midside with the same dose rate as in Experiment 1. After euthanasia, skin punch samples of the injection sites were taken in sequence according to a 3, 5, 10, 20, 30, minute and 1, 2, 4, 6, 8, 10, 12, 17.5, 21 and 24 hour post injection time. The tissues were transferred into pre-chilled plastic vials and frozen immediately until analysis. The sheep used in all experiments were rising two-tooth New Zealand Romney wethers.

Experiment 4: Labelling effect of different BrdU dose-rates

Several sites on a clipped midside patch of a ram were injected in sequence intracutaneously with 0.1 ml BrdU solution of different concentrations (Table 2.1).

Table 2.1 **Concentrations of BrdU solutions**

0.1 ml solutions	total injection loads
0 µg/ml	nil
20 µg/ml	2 µg
50 µg/ml	5 µg
100 µg/ml	10 µg
150 µg/ml	15 µg
300 µg/ml	30 µg
600 µg/ml	60 µg
1 mg/ml	100 µg
3 mg/ml	300 µg

The first set of injections was given 2 hours, the second set 20 minutes before the animal was euthanised. From the injection sites given 2 hours prior euthanasia, skin samples were taken and processed for skin histology and immunocytochemical detection of incorporated BrdU. The labelling effect of different BrdU injection load was determined in histological sections. From the sites of the second set of injections given 20 minutes before euthanasia, skin samples were taken and frozen for subsequent detection of free tissue BrdU.

Histology

Tissue preparation and immunocytochemical detection of incorporated BrdU followed the method described in Chapter 1. Histological tissue sections were screened for the occurrence of label in proliferating bulb cells. To ensure one estimate per bulb, only every tenth section was used for analysis. Criteria for a 'representative' bulb was the presence of a papilla in the section in continuity with the outer connective tissue sheath to indicate that the bulb section was cut close to the median longitudinal axis of the follicle. Only bulbs which met this criteria were chosen for computer aided analysis.

Data collection and evaluation

For the collection of data on follicle bulbs, microscope images were traced with a mouse on a high resolution screen using PC vision systems (PC vision plus frame grabber, imaging, Technology Inc. EC. Gough, Christchurch, NZ.). This set-up produced immediate images of the histological sections on the computer screen. The image analysis software (MARKLINE, Dr. A.J. Hall) then enabled the digitising of points and lines on that same screen and stored the collected datapoints. In this study digitised datapoints were:

I.- number of labelled cells in the follicle bulb

II.- outline of the follicle bulb

III.- outline of the papilla

The labelled cells were identified by eye, using different focal planes to discriminate overlapping nuclei. A minimum of 20 bulbs were measured per sample. Results were then subjected to curve analysis followed by regression analysis.

Permanent records of the digitised follicle bulbs were created for further processing. These parameters were determined beforehand and a program developed to execute the necessary calculations. For this study the parameters were:

I. - number of labelled cells in the follicle bulb as determined by digitised points (**NO**)

II. - three diameter measurements

1) widest diameter across the follicle bulb as determined by the follicle boundary (**DW**)

2) diameter across the follicle bulb at a level which coincides with the top of the papilla (**DP**)

3) diameter across the follicle bulb at a level determined by furthest distal located labelled cell (**DS**)

III. - area measurement of the papilla (**AP**)

IV. - three area measurements of follicle bulb with the distal boundary being determined by the corresponding abovementioned diameter measurements (AW)(AP)(AS)

A detailed description and validation of these measurements are given in Chapter 3, toghether with a figure to illustrate the measurements (Figure 3.1).

High pressure liquid chromatographic (HPLC) detection of free tissue BrdU

Frozen tissue samples were pulverised in liquid nitrogen. The powdered tissue was weighed into chilled tubes and thawed into an equal volume of 10 mM tris-HCl by weight. Samples were vortexed, and stood on ice for 10-15 minutes. Samples were deproteinised by ultrafiltration using 30,000 molecular weight cellulose triacetate centrifuge filters (Pro-X Micro Spin; Lida, Kenosha, WI). Ultrafiltered samples were analyzed using a similar method to that described by Agarwal et al. (1982), on a LC4A Shimadzu HPLC using an ODS RP-18 5 µm column (25 x 4.5 mm; Browntree Labs) flow rate 1.0 ml/min, column temperature 30^{0}C and detected at 254 nm. The mobile phases were 10 mM $NaHPO_4$ pH 4.9 (A) and A plus 60% CH_3CN (B) with 100% A pumped isocratically for 5 minutes, then a linear gradient to 20% B by 20 minutes, then 20% B maintained for a further 5 minutes, then a step to 100% B at 25 minutes maintained to 30 minutes and reequilibrated with A for 10 minutes. Under these conditions, BrdU eluted at 21.0 minutes.

RESULTS

Experiment 1

The photomicrograph in Fig. 2.2 shows the presence of BrdU labelled cell nuclei after a 300 µg injection, confirming the suitability of an intracutaneous injection rather than the infusion methods described in Chapter 1. With these intracutaneous injections, a similar sensitivity of detection of proliferating cells in the follicle bulb is possible. The accumulation of labelled S-phase nuclei in representative follicle bulbs was investigated. The graph in Figure 2.3 shows a plot of the combined mean values and SEM of labelled bulb cells plotted against time after BrdU injection.

Since the frequency distribution of cell nuclei counts in longitudinal bulb sections did not deviate from Gaussian normal frequency distribution, numbers are represented as means. It showed that the administrated BrdU was incorporated immediately into the DNA of proliferating cells since after 5 minutes labelled nuclei were already detectable.

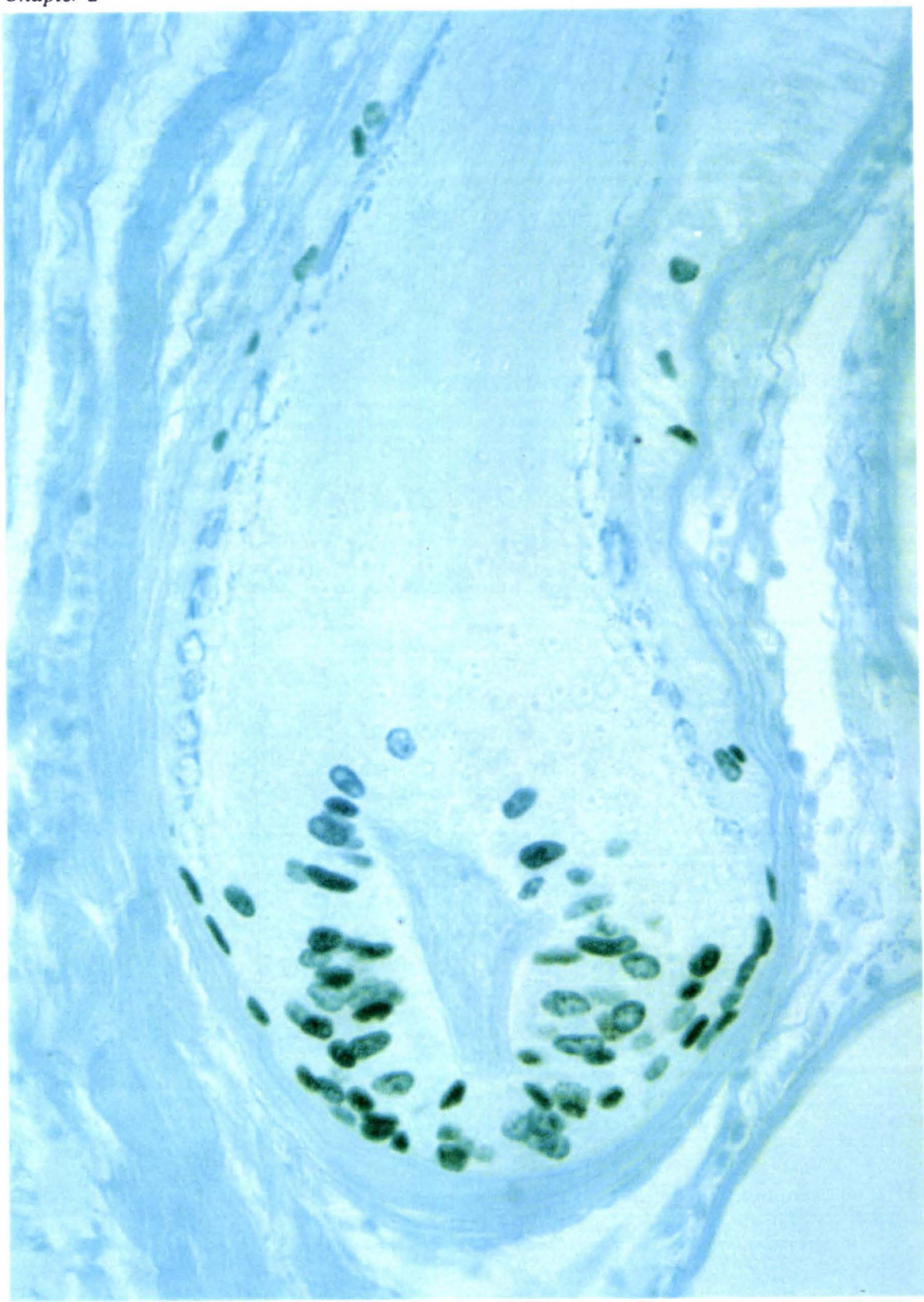

Figure 2.2 **Longitudinal section through the lower parts of a wool follicle. Counterstain: 0.1% acetic light green. Magnification: 571x. Labelling effect detected with immunostain biotin-streptavidin method 2 hours after a 300 µg i.c. BrdU injection load**

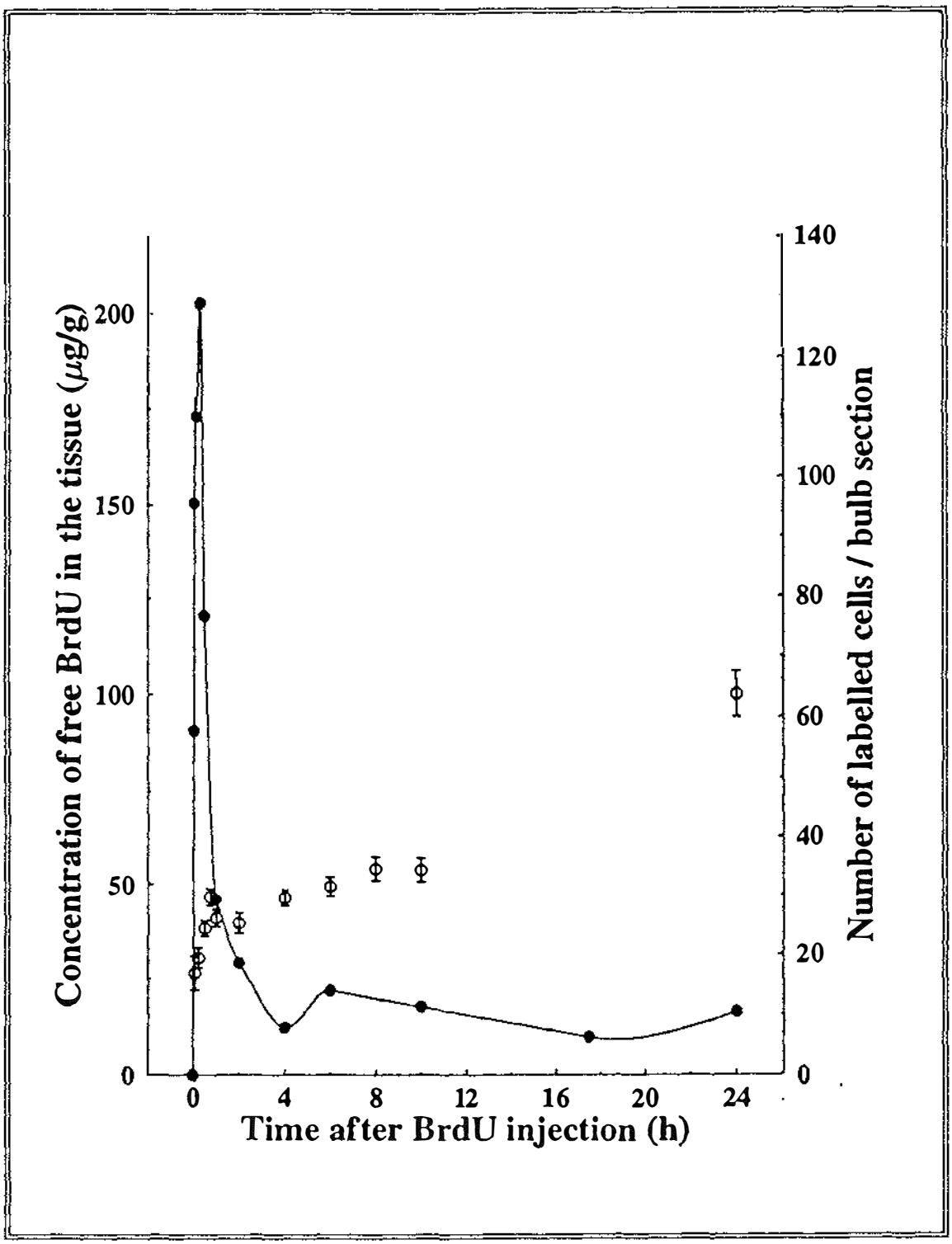

Figure 2.3 **Number of BrdU labelled cells in follicle bulb sections and concentration of free BrdU in the tissue (µg/g tissue) after a 300 µg i.c. injection. Free BrdU in the tissue (•); number of BrdU labelled cells (○). Error bars indicate the SEM.**

The plotted data show an extremely rapid increase from the origin to the first dataset at 5 minutes. From 5 minutes through to 24 hours the number of labelled cells increased more gradually in a linear fashion. Alternative curves shapes (exponential, logarithmic) were also fitted to the data but linear regression gave the best fit. The data were regressed with and without the 24 hour samples, in order to establish the relative influence of these data on the slope of the regression. Because omitting 24 hour samples made only a slight difference to the regression coefficients, these data were included in the final regression (with the 24 hour data: $y=34.43+2.63(\pm0.25)*x$, $r=0.96$; without the 24 hour sample: $y=35.74+2.14(\pm0.15)*x$, $r=0.83$). Mean values and correlation coefficients are given in the Appendix (page: A.6 and A.7).

The relationship of the number of labelled cell nuclei appeared to yield sufficient information to justify predictions on their increase in number with time. The measurements on follicle dimensions (diameter and area) were then used for regression analysis in combination with counts of labelled nuclei, in order to evaluate whether there was any closer association when including any of the follicle dimensional parameters and the time at which the samples were taken, and whether it is possible to make predictions on one or more of those parameters with time.

Plotted data for the Y-axis were:

I. - number of labelled cells per diameter **(DW); NO/DW**

II. - number of labelled cells per diameter **(DP); NO/DP**

III. - number of labelled cells per diameter **(DS); NO/DS**

IV. - number of labelled cells per corrected area **(AW);**
NO/AWC-PA

V. - number of labelled cells per corrected area **(AP);**
NO/APC-PA

VI. - number of labelled cells per corrected area **(AS);**
NO/ASC-PA

(corrected area= bulb area minus papilla area)

Table 2.2 **Regression coefficients for the number of labelled bulb cells corrected for three different diameter measurements regressed against time**

b : intercept

a : slope

DW : diameter through the widest part of the follicle bulb

DP : diameter through the bulb at the top of the papilla

DS : diameter through the bulb at the furthest distally located labelled cell in the follicle

No : number of labelled cell nuclei in the follicle bulb section

regression equation: y = b + a * x												
sheep No	No/DW versus time				No/DP versus time				No/DS versus time			
	b	a	STD of a	R-sq %	b	a	STD of a	R-sq %	b	a	STD of a	R-sq %
1	0.276	0.0156	0.0008	67.1	0.281	0.0158	0.0008	65.7	0.0070	0.0192	0.0009	70.3
2	0.301	0.0144	0.0011	48.9	0.313	0.0146	0.0011	48.6	0.3056	0.0164	0.0012	49.9
3	0.213	0.0151	0.0024	24.7	0.214	0.0154	0.0025	24.4	0.2133	0.0153	0.0024	25.2
4	0.213	0.0104	0.0016	31.7	0.221	0.0113	0.0012	33.3	0.2290	0.0052	0.0059	01.3
5	0.271	0.0161	0.0031	22.4	0.284	0.1491	0.0033	18.8	0.2780	0.0156	0.0031	21.4
6	0.330	0.0079	0.0032	02.8	0.338	0.0068	0.0033	02.5	0.3363	0.0071	0.0032	02.8

Table 2.3 **Regression coefficients for the number of labelled bulb cells corrected for three different area measurements against time**

aw : follicle bulb area under dw, corrected for papilla area

ap : follicle bulb area under dp, corrected for papilla area

as : follicle bulb area under ds, corrected for papilla area

No : number of labelled cell nuclei in follicle bulb section

regression equation: y = b + a * x												
sheep No	No/aw.corr.versus time				No/ap.corr.versus time				No/as.corr.versus time			
	b-intercept	a-slope	STD of a	R-sq %	b-intercept	a-slope	STD of a	R-sq %	b-intercept	a-slope	STD of a	R-sq %
1	0.00437	$0.3034x10^{-4}$	$0.229x10^{-5}$	48.5	0.00449	$0.2520x10^{-4}$	$0.221x10^{-5}$	41.1	0.00473	$0.137x10^{-5}$	$0.114x10^{-5}$	00.8
2	0.00381	$0.2572x10^{-4}$	$0.239x10^{-5}$	38.0	0.00443	$0.2763x10^{-4}$	$0.246x10^{-5}$	39.8	0.00471	$0.684x10^{-5}$	$0.137x10^{-5}$	11.6
3	0.00347	$0.3157x10^{-4}$	$0.696x10^{-5}$	14.6	0.00433	$0.3109x10^{-4}$	$0.982x10^{-5}$	07.7	0.00427	$0.193x10^{-4}$	$0.567x10^{-5}$	08.7
4	0.00359	$0.1717x10^{-4}$	$0.455x10^{-5}$	13.9	0.00446	$0.4732x10^{-4}$	$0.122x10^{-4}$	14.5	0.00496	$0.126x10^{-4}$	$0.335x10^{-5}$	13.9
5	0.00498	$0.2788x10^{-4}$	$0.728x10^{-5}$	13.5	0.00753	$0.1808x10^{-4}$	$0.138x10^{-4}$	01.8	0.00669	$0.139x10^{-4}$	$0.668x10^{-5}$	04.4
6	0.00468	$0.6380x10^{-5}$	$0.500x10^{-5}$	01.0	0.00566	$0.6150x10^{-5}$	$0.701x10^{-5}$	00.5	0.00595	$0.591x10^{-5}$	$0.534x10^{-5}$	00.7

By correcting the number of labelled cells for any of the three diameters (Table 2.2) or any of the area measurements (Table 2.3) no improvement in relationship with time was found.

The rate of accumulation of labelled cells was calculated from the difference of the combined means of all sheep. Special emphasis was put on the time period from 2 to 6 hours since this interval was chosen for a field trial described in Chapter 4.

Table 2.4 **Mean number of labelled follicle bulb nuclei in 6 sheep after 2 and 6 hours of i.c. BrdU injection. Analysis of variance for significance of the difference in accumulation of labelled cells from 2 to 6 hours and rate of accumulation per hour**

	MEAN	SEM	F	p	N
2 hours	39.87	3.77	2.26	0.164	12
6 hours	49.50	5.19			
rate h^{-1}	**2.41**	**0.91**			

The increase in labelled cells did not reach significance. Nevertheless an increase of 2.41 cells/hour between 2 and 6 hours after i.c. injection of BrdU was observed (Table 2.4). Compared to the regression equation given for Table 2.3, the calculated rates approximate the slope values.

Experiment 2

The injection sites in Experiment 2 showed a clear, strong positive labelling reaction. Samples taken from sites in a radius of 5 mm away from the outer edge of the skin samples over the BrdU inoculation frequently showed a staining reaction. At all other sites no BrdU incorporation could be detected, demonstrating that the labelling effect is wholly confined to a radius less than 20 mm from the injection site.

Experiment 3

In this experiment, the changes with time in the concentration of free BrdU which was not incorporated into cellular DNA were measured. These results reflect the dilution rate of BrdU dependent on the metabolic activity of the tissue and the proliferation rate of the cell population. Figure 2.3 on page 2.10 shows the plotted data for tissue levels of free BrdU as measured by HPLC. The level of free BrdU in the injected skin tissue reached a peak 20 minutes after injection. The concentration declined exponentially over the next several hours and reached a low of about 10% of the peak level by 12-24 hours.

Experiment 4

Figure 2.4 shows photomicrographs of some examples of detectable BrdU-labelled follicle bulb cells after injection of BrdU-solution of different concentrations. Amounts as low as50µg still achieved a satisfactory immunocytochemical labelling reaction. 20µg injection loads showed a fading colour reaction in occasional bulbs, while the majority of bulbs in the examined sections showed no reactivity. Free BrdU was detectable 20 minutes after injection in samples injected with 15 µg BrdU or more (Figure 2.5). All lower injection loads had a free pool less than the limit of the method of HPLC detection, which was 9 µg/g tissue.

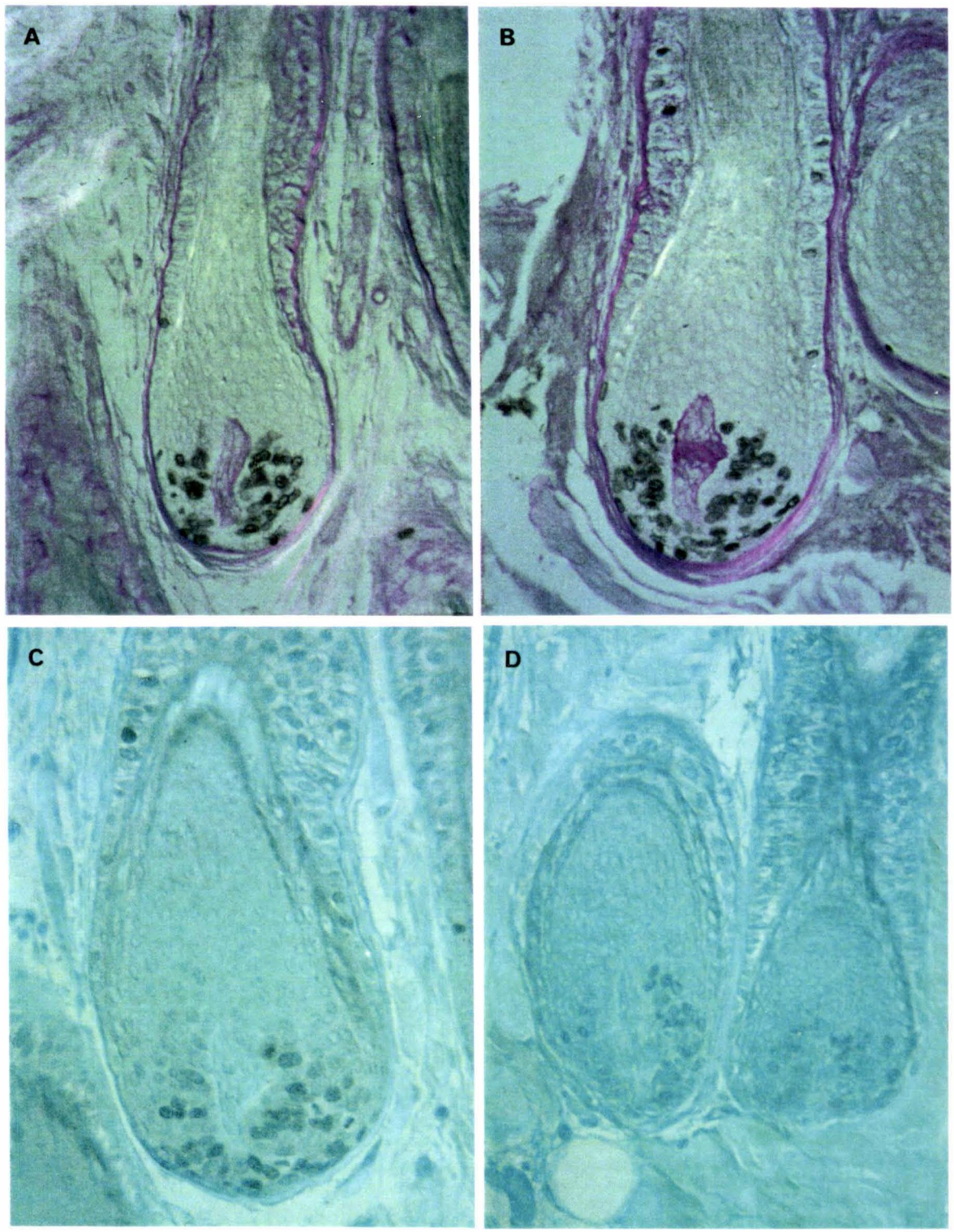

Figure 2.4 **Longitudinal sections through the lower parts of follicle bulbs showing BrdU-labelled cells after different BrdU injection loads.** **(A)** PAS stain. Magnification: 266x. Injection load: 1000 µg. **(B)** PAS stain. Magnification: 266x. Injection load: 600 µg. **(C)** 0.1% acetic light green. Magnification: 288x. Injection load: 50 µg. **(D)** 0.1% acetic light green. Magnification: 226x. Injection load: 20 µg. **A,B, and C show a strong staining reaction, D shows almost no BrdU incorporation**

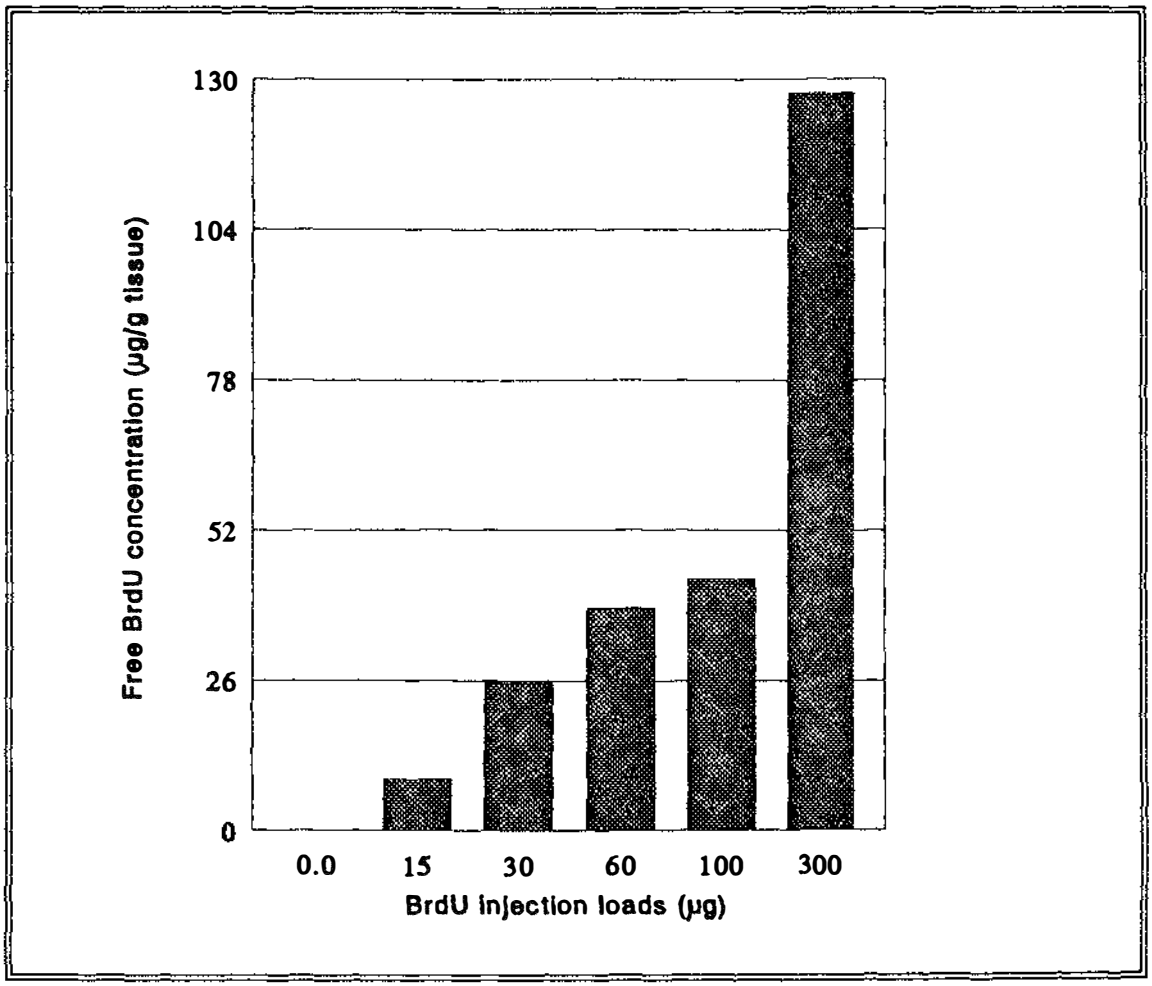

Figure 2.5 **Concentration of free BrdU in the tissue (µg/g tissue) of different BrdU injection loads, 20 minutes after injection, detected by HPLC. (y=6.49+0.41(±0.027)*x, r=0.99). Only injection loads detectable with the HPLC method are given.**

DISCUSSION

Toxicity

After investigating the possibility of vascular infusion of BrdU into isolated midside skin patches of sheep and detecting the labelled follicle bulb nuclei immunocytochemically as outlined in Chapter 1, the present study describes an intracutaneous administration of BrdU into the skin of sheep. The i.c. injection methods resulted in dark stained nuclei in histological sections, using the same detection method as described in detail in Chapter 1. Intracutaneous administration of BrdU is preferable to infusion into isolated skin patches, because it avoids surgically modifying the animals. It is preferable to i.v. administration and more appropriate for field studies, because of the ease of injection compared to full i.v. application.

A recent study with BrdU (Adelson et al., 1991) has associated intravenous administration of BrdU with high levels of wool fibre abnormality. They suggest that ^{3}H-thymidine may be superior to BrdU as a label for tracking wool follicle cells with time. This result may, however, be due to systemic toxicity. Prolonged i.v. infusion of BrdU causes severe toxic side effects in sheep with the epithelial lining of the digestive tract immediately affected, followed by severe diarrhoea and nutritional malabsorption (V.J. Westlake and M.J. Birtles, personal communication). Thus, whole body infusion of BrdU, especially in high doses, by perturbing normal gut structure and function, can result in lowered nutrient supply, including supply of amino acids, to all body tissues including the skin. Lysine and the sulphur containing amino acids are the first limiting amino acids for wool protein production and their omission from the diet has a profound effect on wool growth through reduction in the strength of wool fibres (Reis, 1987). Lysine is also known to be an essential amino acid for the inner root sheath (IRS) development (Reis and Tunks, 1978). Chapman et al. (1983) observed reversible abnormalities in follicle and fibre in pre-ruminant lambs fed with a deficient level of dietary lysine. These aberrations included incomplete and retarded keratinization associated with fibres lacking distinguishable cuticle patterns. On the other hand, a surplus of lysine in the diet hastened the hardening of the IRS. Since the integrity of the IRS is ultimately responsible for the expression of fibre cuticular scale pattern, any abnormality at the IRS level subsequently affects fibre surface morphology.

The abnormal features observed in fibres apparently caused by toxic effects of whole body infusion of BrdU/FdU (Adelson et al., 1991) are strikingly similar to the abnormalities produced by lysine deficiency (Chapman et al., 1983). Cytotoxic effects of BrdU can be partially explained by gut perturbations rather than a direct effect on wool follicle cell function

itself. This would be avoided by local BrdU administration. In addition, Hynd (1989b) postulated that cysteine in the fluid spaces surrounding the follicles might alter cell differentiation by influencing gene transcriptional processes such that the high-sulphur-paracortex would only be coded for in the presence of high levels of cysteine. This hypothesis could well explain the decreased expression of paracortex specific proteins in the fibre observed by Adelson et al. (1991), if circulating cysteine levels are also depressed through gut absorbtion abnormalities. One should be aware of the potential cytotoxic effects of BrdU, but as well, the cause of aberrations in fibre morphology associated with BrdU should be interpreted cautiously.

In none of the sections studied here was any cell malfunction or morphological disturbance, such as apoptosis or pyknosis observed. These samples were only taken up to 24 hours after BrdU administration and a longer period of exposure may be necessary to finally establish whether the abnormalities associated with whole body administration of BrdU are also a problem with i.c. administration. Intracutaneous BrdU administration gives the minimum load to label dividing cell populations, while avoiding systemic toxicity.

Time course labelling

Hynd et al. (1986) indicated that linear accumulation of metaphase nuclei must occur if proliferating cells are to be quantified. To complement data on increases of labelled cells in estimates of cell kinetic parameters, information on the concentration of free BrdU after injection is necessary to permit an accurate quantification of the numerical changes in the underlying cell population.

Concentration changes of non-DNA bound BrdU are closely linked to the metabolic clearance processes from the tissue as well as to the proliferative activity of the cell, both contributing towards dilution of the initial BrdU concentration. The shape of the curve in Figure 2.3 shows a rapid increase of the number of labelled cells immediately after a single intracutaneous injection of 300 µg BrdU. This is due to the presence of numerous cells in S-phase at the time of injection, ready to incorporate BrdU, as well as a small number of cells entering S-phase over the next few minutes. At this stage, sufficient BrdU is available for all proliferating cells to be detected. The later, linear, increase of labelled cells can be affected by several components, intrinsic to the tissue or characteristic of the agent introduced. The actual proliferation rate of the population can only be fully represented by this linearity if sufficient label is available to be incorporated. The proportion of labelled cells will increase each time a labelled cell divides, and decrease with each division of an unlabelled cell.

Label pharmacokinetics

After 300 µg intracutaneous injection, the free BrdU took up to 20 minutes to reach a peak. This delay suggests that the i.c. injection was deeper than the level of sampling, and that some time was necessary for diffusion of the free BrdU. Sample variation might be reduced in future studies by using shallower injection depths.

Even after 24 hours, the level of free BrdU was around 20 µg/g tissue, which was approximately 10% of the HPLC measured 20 minute peak. The concentration study in Experiment 4 resulted in the observation that even small amounts (5 µg) of label at injection are detectable immunocytochemically with a satisfactory colour reaction, even though the level of free BrdU 20 minutes after a 5-15 µg injection was below the limits of the HPLC detection system. After a single 150-300 µg i.c. injection, label is present in the tissue for a 24 hour period in a concentration (10-20 µg/g tissue) able to label proliferating cells sufficiently. Consequently, this mode of injection does not behave as a local pulse but rather as a constant infusion over 24 hours in terms of availability of label.

Based on a model for epidermal growth of an asynchronous amplifying scheme for cell populations (Potten, 1978), cells are capable of undergoing up to 3 subsequent divisions. This, in turn, is well within the limits of BrdU dilution due to multiple mitosis (Wynford-Thomas and Williams, 1986). Thus, linear accumulation of labelled cells represents all proliferating cells which undergo S-phase in this 24 hour period. This includes cells which have not been labelled before as well as already labelled cells which have undergone one mitosis and enter their second S-phase during the recorded time span.

The growth fraction is the proportion of cells proliferating at any one time, assuming that some cells in the total population are noncycling cells, and is represented by the maximum number of cells labelled by continuous exposure (Baserga, 1989). The slow clearance rate of the label in skin tissue, in combination with an extremely sensitive method for labelling and detection of BrdU, means that i.c. injection is particularly suitable for the continuous exposure of tissue for cell growth studies such as the determination of growth fractions in the germinative tissue of the follicle bulb.

The levels of i.v. administration used by Adelson et al. (1991), Holle and Birtles (1990) and Hynd and Everett (1990) were 1,000 fold the amounts required for intracutaneous administration. Intravenous administration labels all body tissues simultaneously, and so is desirable for inter-tissue comparisons. However, when specific studies of skin or wool are required, the localised dose, the ease of application and the repeatability of measurements makes i.c. administration better from an ethical, practical and mensural standpoint.

Chapter 3

The Relationships between Cell Proliferation and various Follicle Bulb and Dermal Papilla Measurements, Compared between Seasons and between different Levels of Follicle Output

INTRODUCTION

The anagen follicle in sheep, although continuously growing fibre, is subject to cycles of activity decline, brought about by seasonal, nutritional and/or genetical influences, measurable by the decrease in fibre length growth rates and fibre diameter (fibre volume output). Little information is available on the influence on fibre production of dimensional variations of the follicle at bulb level and dimensional relationships of follicular structures. Contributions have been made by VanScott (1965) and Orwin (1989). There are difficulties in correctly defining the dimensions to be measured, especially for the bulb area and germinative region, as already outlined in the general Introduction (page: I.19).

The key questions which need to be addressed are:

1) Do dimensional relationships between follicle structures at the bulb level exist, such as between

   a) the area and diameter of the follicle,

   b) the length and area of the dermal papilla,

   c) or between these and the number of dividing cells?

2) Are these relationships influenced by genotype or season as indicated in follicle size and fibre production variations?

3) Are the differences in the strength of the relationship of closely associated parameters real, or dependent on artificial definitions? How well do different measurements of the same structure reflect functional changes in it?

4) What is the dimensional relationship of cortex to IRS, and how is it related to wool production, bulb dimensions and cellular events in the bulb?

5) Considering total cell number in the follicle bulb:

a) how much does a cell number change account for size changes in the follicle bulb?

b) how compatible are cell number counts in the bulb area to bulb area and diameter measurements?

c) how is the total number of cells related to the number of dividing cells?

Follicle size

Follicle size is believed to be related to fibre diameter, in so far that larger follicles generally produce coarser fibres (Burns and Clarkson, 1949) and fine-woolled breeds tend to have follicles shorter in length and narrower in diameter (Henderson, 1965; Chapman and Gemmell, 1971). The follicle can be divided into two components, the bulb and the dermal papilla.

Dermal papilla

According to studies by Billingham and Silvers (1965), the dermis in mammals controls the histological character of the overlying epidermis. The dermal papilla forms the dermal portion of the follicle and also appears to play an essential role in hair (Hashimoto and Shibazaki, 1976) and wool growth (Rudall, 1956). The importance of the role of the dermal papilla has been stressed already in the general Introduction (page: I.16). Its dimensions are related to bulb size, and hence in turn to fibre dimensions. Its height is subject to seasonal changes in sheep (Rudall, 1956) being shorter during winter. In rodents, dermal papilla height varies in relation to the hair cycle (Cohen, 1965).

Which papilla property influences bulb or fibre dimensions most is unclear. Burns and Clarkson (1949) found no relationship between the papilla shape and the fibre grown, although positive correlations between papilla volume and surface area and fibre dimensions have been claimed (Burns and Clarkson, 1949; Auber, 1952; Rudall, 1956; Henderson, 1965).

The size of the dermal papilla appears to influence the mitotic activity in the hair bulb (VanScott and Ekel, 1958) as well as the distribution of mitotic figures in the bulb (VanScott et al. 1963). As outlined in the general Introduction (page: I.22), the dermal-epidermal contact area plays a crucial part in altering kinetics of epithelial cell populations. Increased proliferation can be brought about through elongation of this connection line (VanScott and Ekel, 1963). Alterations in length and width of the dermal papilla, both affecting overall area, will

have implications for the surrounding bulb. The underlying basis of the relationship between the dermal papilla and bulb size is believed to be the size of the dividing cell population associated with the papilla surface (Henderson, 1965). This is confirmed for epithelium during conditions of enforced growth, such as psoriasis or benign epidermal hyperplasia (VanScott, 1965).

Burns and Clarkson (1949) concluded that regular dimensional relationships are characteristic of follicles during optimal or near optimal wool production, but that they become disturbed when follicle activity is not optimal. In their study, dermal papilla size was measured in longitudinal follicle bulb sections by length, and its area was determined by tracing its outlines. Other follicle dimensions were directly related to these measurements.

Follicle bulb

In addition to its relationship to the dermal papilla, bulb size is highly correlated with fibre diameter and volume (Rudall, 1956; Schinckel, 1961; Henderson, 1965; Wilson and Short, 1979), bulb size in these studies being used to gain information on the size of the population of dividing cells. Williams and Winston (1987) showed in two Merino lines selected for high and low fleece production that although follicle bulbs had different areas of mitotically active cells, they had the same mean diameter.

Bulb size, in the same way as fibre dimension, can be changed by nutrition and season (Rudall, 1956; Henderson, 1965; Short et al., 1965; Wilson and Short, 1979). Nutritional levels can affect both mitotic activity and bulb size (Schinckel, 1962; Wilson and Short, 1979; Hynd et al., 1986).

These studies demonstrate the difficulty of drawing conclusions about underlying morphological mechanisms. This is especially so if dimensional measurements are not standardised or at least clearly defined beforehand.

Relationships between different follicle bulb measurements, and size of the germinative tissue zone

The exact boundary of the proliferative bulb tissue, as already mentioned in the general Introduction (page: I.19), is not clear. In order to determine the existence of a mechanism influencing area size shifts that responds to seasonal, nutritional and/or genetic influences, there should be independent criteria to judge the boundaries of the germinative region. Also, no study so far has validated the relationship between bulb area and bulb diameter, even though both

measurements are used concurrently (Williams and Winston, 1987). Especially if the size of the germinative region is to be determined, it appears to be insufficient to measure only bulb diameter and extrapolate the results (Short et al., 1965).

In order to determine the proliferative activity of the germinative cell population in the follicle bulb, Short et al. (1965) counted mitotic as well as interphase nuclei, as well as bulb width and area measurements, but no relationship was established between numbers of mitotic nuclei, total cells and area measurements. The confusion caused by using different definitions in order to derive variables such as turnover time and generation time, was discussed by Epstein and Maibach (1965). They stated that, depending on where the boundary for the germinative tissue region is drawn, either not all the cells are undifferentiated or the area does not contain the total number of the viable cell population. Consequently, calculated turnover times might be too long for the germinative layer or too short for the total population. The complex organisation of the bulb matrix area, in combination with technical problems concerning stereomorphological measuring methods, makes estimation of any sort become rather difficult. The variable input of cells into the IRS and cortex at different times and the possibility of size shifts in the germinative population (Priestley and Rudall, 1965) add to the problem.

Relationships of cortex and IRS

A number of studies have implicated that the relative distribution of IRS cells and cortex might influence total wool production. Butler and Wilkinson (1979) determined a "production ratio" from cross-sectional area measurements of fibre and fibre plus IRS, noting a relationship to the efficiency in wool production (g wool/100 g digestible dry matter in the diet (DDM)) in Corriedale sheep. For the Merino, Short et al. (1965) found that the IRS accounted for 50-55% of the total area occupied by IRS and fibre. This was measured in a segment of longitudinal follicle sections above the dermal papilla. Factors such as nutrition (Short et al., 1965), genotype (Wilson and Short, 1979) and season (Priestley, 1967a) have been stated to influence the distribution of cells into fibre and IRS. Furthermore, findings by Straile (1965) that the cross-sectional shape of fibre and IRS is maintained despite changes in fibre shape at different stages of hair growth, imply variations in the cell distribution between the two follicle structures.

Hynd (1989a) noted a cortex proportion (as part of IRS plus cortex) of 0.46, which was unaffected by diet but was poorly related to the proportion of dividing cells entering the fibre ($r=0.28$, $P<0.33$). Also, he noted a wide within sheep variation for cortex proportion which increased with improved nutrition ($p=0.01$). He suggested that a genetic component determines the proportion of dividing cells that contributes to the fibre cortex.

Total number of cells in the follicle bulb

Studies by Schinckel (1961, 1962), Fraser (1965), Short et al. (1965), Wilson and Short (1979), Hynd et al. (1986) and Hynd (1989a) and Hynd and Everett (1990), included estimates of total cell numbers in the follicle bulb of Merinos. The bulb area or germinative tissue zone in which cells were counted was defined using various criteria. An estimate of a total number of 300 to 1200 cells has been given (Hynd et al., 1986) with a 5% proportion of cells undergoing mitoses every hour. Sheep fed on high protein diets (Hynd, 1989a) showed an increase of the germinative tissue region and in the number of mitotic cells, but with the overall effect being an unaltered turnover time. Both traits were highly related (r=0.9).

Colebrook et al. (1988) studied the relative importance of cell number to cell size in determining the mass of organs and tissues in sheep. Cell number was largely responsible for differences in organ mass. If the follicle follows this pattern, a numerical increase of its cells will form a larger proliferating population. This new population (once it is established) produces at a similar rate as before.

In this chapter, several measurements of bulb and papilla dimensions are made in an attempt to determine which measurements are the best indicators of wool production changes. Also, the relationship of follicle bulb and dermal papilla dimensions with the number of total and proliferating cells in the lower area of the follicle bulb has been investigated.

Follicles of Romney sheep on two genetically different levels of wool production, examined during periods of low (winter) and high (summer) fibre output, form the experimental model that relates morphological changes of follicles to functional variations in their activity. The main production comparisons between the two selection lines and seasons in follicle and fibre characteristics are presented in Chapter 4.

MATERIALS AND METHODS

Details on skin sampling and main trial comparison are given in Chapter 4 (page: 4.4). Several measurements of follicle and dermal papilla dimensions were made in order to assess which of these, or combinations of these, would discriminate best between the two genetically different lines, under seasonal influence. The number of observations for the follicle population used for this study is that listed in Table 4.13 in Chapter 4 (page: 4.15). The following lists the parameters measured, and their definition.

The digitised parameters were:

I. - number of labelled cells in the follicle bulb (**NO**)

II. - diameters across the follicle bulb

1. diameter across the widest part of the bulb (**DW**)
2. diameter across the bulb intersecting with the top of the dermal papilla (**DP**)
3. diameter across the bulb through the most distal labelled bulb matrix cell (**DS**)
4. diameter across the first cell layer on top of the dermal papilla (Fraser, 1965), for the distal boundary for the germinative region of the bulb (**DF**)
5. diameter through the fibre cortex at its origin about 1 cell layer distal to the papilla apex (**DC**)
6. diameter through the cortex plus IRS at their origin 1 cell layer distal to the papilla apex (**DI**)

For points 5 and 6, their origin was determined by the observation of colour intensity changes in the counterstain, which has been described in Chapter 1.

III. - area of the dermal papilla (**PA**)

IV. - length of the dermal papilla, as determined from its apex to its joining point with the connective tissue sheath (**PL**)

V. - follicle bulb areas

1. area corresponding to diameter DW (**AW**)
2. area corresponding to diameter DS (**AS**)
3. area corresponding to diameter DP (**AP**)
4. area under a curved line around the papilla one cell layer distal to its apex and intersecting either side with the bulb wall at a level where the differentiation of the IRS becomes visible (Short et al., 1965) (**AC**)

All of these parameters are shown in Figures 3.1 and 3.2.

Details on the image analysis are given in Chapter 2 (page: 2.7). Concepts on follicle bulb area and diameters are stressed in the introduction.

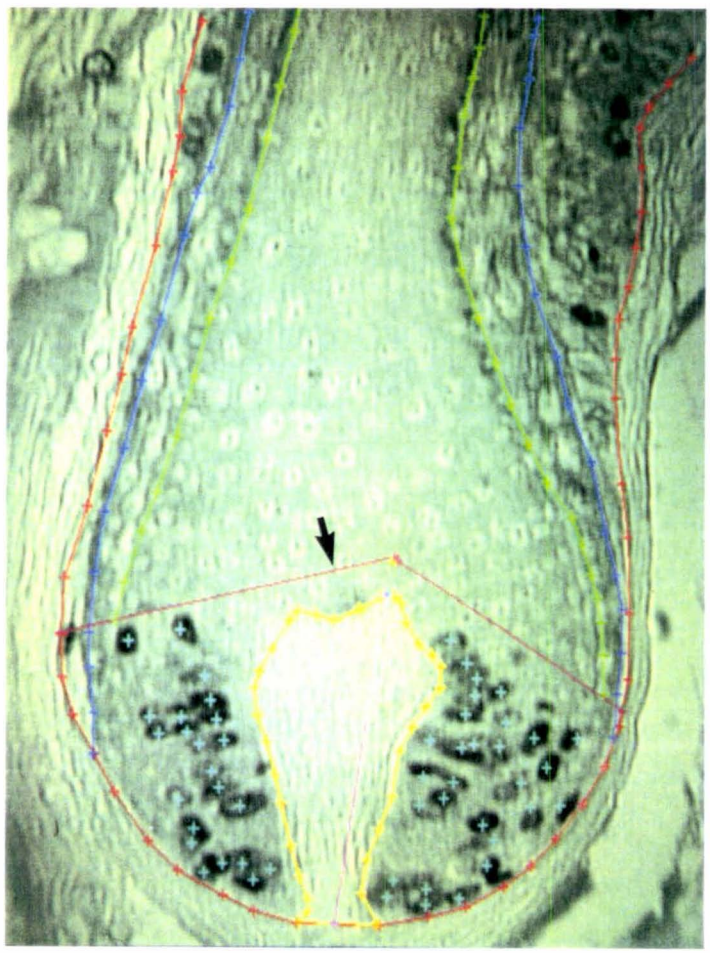

Figure 3.1 **Photomicrograph of a longitudinal section through a follicle bulb projected on a computer screen, showing the different measurements made with the image analysis software.** *red line*: ORS/connective tissue boundary; *blue line*: ORS/IRS boundary; *green line*: IRS/cortex boundary; *yellow line*: dermal papilla area; *pink line*: dermal papilla length; *blue crosses*: BrdU-labelled cells; *arrow*: upper boundary for germinative tissue zone AC

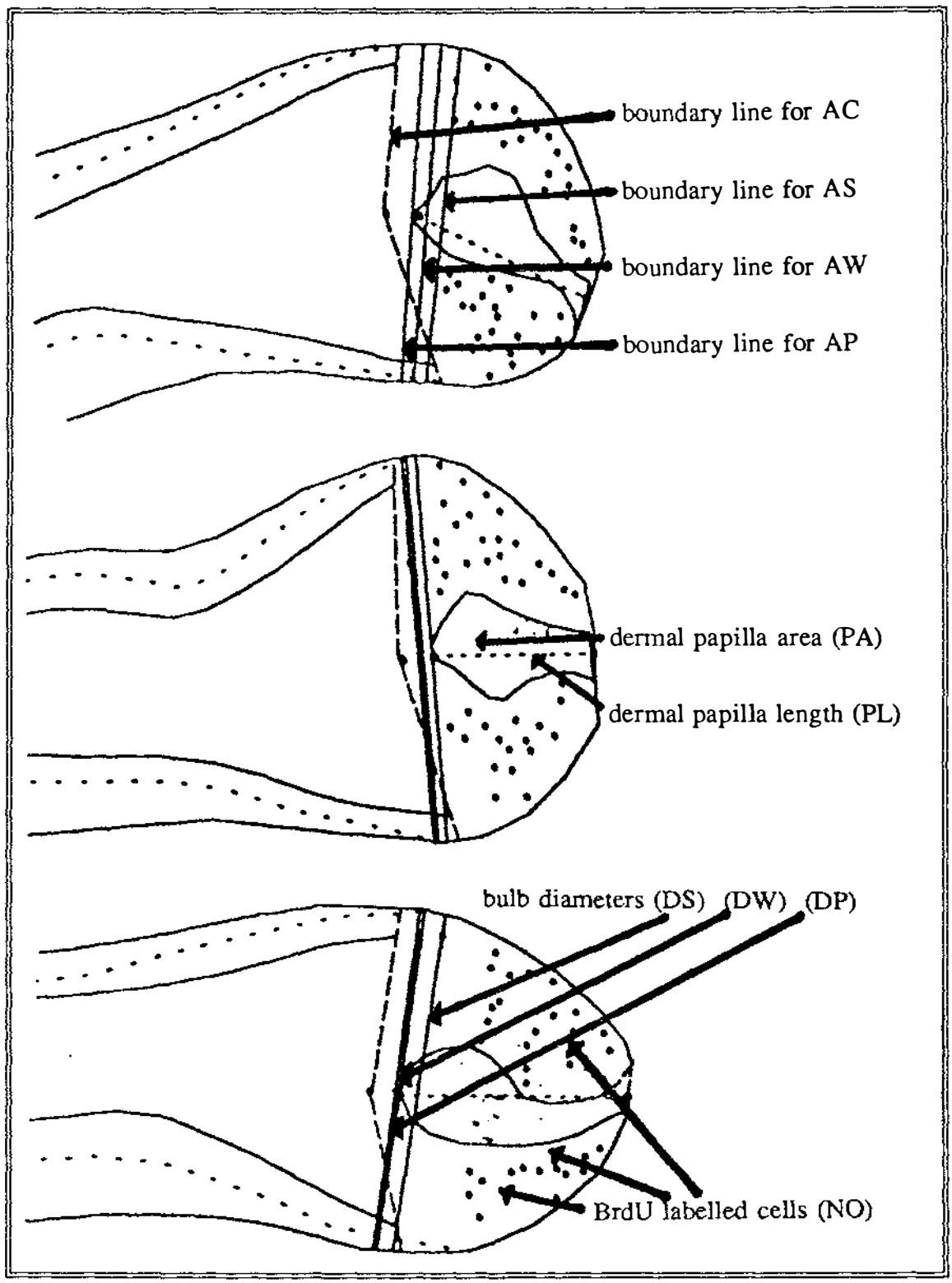

Figure 3.2 **The various measurements made on longitudinal follicle bulb sections, each shown on three examples, but each is labelled on only one of the follicles shown**

In order to show general patterns in morphological mechanisms, phenotypic extremes in the selection lines were chosen, to minimize interference of errors introduced by variations of individual animals overriding the genotypic effect of the selection lines. A small subset of follicle data (Number of observations: Table 3.8) from each selection line was taken for each month, selecting the follicle population with the highest mean number of proliferating cells for FWT sheep and with the lowest mean number of proliferating cells for CLT sheep respectively.

In this subset, the number of unlabelled cells in longitudinal bulb sections was counted under the light microscope in order to determine:

1. total cell numbers in the bulb area
2. relationships of total numbers to proliferating cells
3. relationships of total cell numbers to follicle bulb and dermal papilla dimensions

The number of proliferating cells was related to two areas, two diameters and to the dermal papilla length to create density ratios (derived variables). The bulb areas were corrected to remove the effect of the dermal papilla area.

Derived variables were:

- NO/corrected area AS [AS-PA] **(NO/ASC)**
- NO/corrected area AP [AP-PA] **(NO/APC)**
- NO/bulb diameter DP **(NO/DP)**
- NO/bulb diameter DS **(NO/DS)**
- NO/dermal papilla length PL **(NO/PL)**

Statistical analysis

Canonical discriminant analysis was used to determine the correlation coefficients between parameters in June, August and November respectively. The correlation coefficients were expressed as pooled within-class correlation coefficients in order to eliminate the between group effects. Analysis of variance was used on individual parameters for each month.

Derived density variables were subjected to general linear model procedures to test for month, group and month x group interactions (SAS Institute Inc. Cary, NC, USA.).

General linear model procedures are used as analysis of variance models to analyze unbalanced data using dummy or indicator variables in the regression model, because otherwise the direct computation of sums of square fails. Least square means are produced which are adjusted means, and are different from simple unadjusted means (Crum, 1986).

RESULTS

Diameters

The bulb diameters (DW, DP, DS, DF) proved to be highly correlated (r=0.9) during June, August and November respectively (Table 3.1; A,B,C).

Correlation of DC and DI with other diameters was slightly weaker (r=0.8) in August and in June (r=0.7). A significant change became obvious in November when DC dropped to r=0.6 and DI to r=0.2 in their correlation with other diameter measurements. DC and DI were correlated with r=0.19 in November.

Table 3.1 **Correlation coefficients for diameter measurements (pooled within-class correlation coefficients)** All are highly significant ($p < 0.001$)

JUNE

	DW	DP	DS	DF	DI	DC
DW	-	0.989	0.992	0.989	0.716	0.751
DP		-	0.987	0.999	0.723	0.751
DS			-	0.987	0.711	0.751
DF				-	0.723	0.758
DI					-	0.578
DC						-

AUGUST

	DW	DP	DS	DF	DI	DC
DW	-	0.991	0.994	0.991	0.803	0.861
DP		-	0.990	0.999	0.814	0.861
DS			-	0.991	0.803	0.861
DF				-	0.814	0.876
DI					-	0.834
DC						-

NOVEMBER

	DW	DP	DS	DF	DI	DC
DW	-	0.977	0.981	0.976	0.20	0.665
DP		-	0.972	0.999	0.193	0.650
DS			-	0.972	0.210	0.657
DF				-	0.194	0.649
DI					-	0.192
DC						-

Bulb areas

Although bulb areas are not as highly correlated to each other as are diameters, they show a constant relationship over the three months of observation. AP to AS expressed the closest relationship, followed by AW to AP and AS (Table 3.2; A,B,C).

Table 3.2 **Correlation coefficients of bulb area measurements.**

All are highly significant ($p < 0.001$)

JUNE

	AW	AP	AS
AW	-	0.604	0.589
AP		-	0.721
AS			-

AUGUST

	AW	AP	AS
AW	-	0.621	0.588
AP		-	0.721
AS			-

NOVEMBER

	AW	AP	AS
AW	-	0.601	0.558
AP		-	0.715
AS			-

Follicle bulb diameters to bulb areas

The bulb area measurements should be strongly related to their corresponding diameters. It was assumed that, by definition, the bulb area measurement would be strongly related to their corresponding diameters, for example area AP is under the line across the top of the dermal papilla. This line coincides with diameter DP.

Table 3.3 gives the correlation coefficients for diameters and area measurements. The corresponding measurements did not show the strongest relationship, but there appeared to be a constant overall relationship of approximately r=0.7. The only exceptions were seen with AW to DW, DP and DS. The relationship within areas and between areas and diameters seemed equally strong without striking differences between seasons.

Table 3.3 **Correlation coefficients for area measurements to their corresponding diameter measurements.** All are highly significant ($p < 0.001$). Coefficients of physically linked parameters (i.e.AW-DW) are shown in grey filled cells.

JUNE

	DW	DP	DS
AW	0.613	0.591	0.575
AP	0.712	0.695	0.713
AS	0.701	0.695	0.715

AUGUST

	DW	DP	DS
AW	0.685	0.666	0.670
AP	0.747	0.726	0.741
AS	0.736	0.717	0.722

NOVEMBER

	DW	DP	DS
AW	0.627	0.580	0.547
AP	0.687	0.692	0.671
AS	0.683	0.659	0.686

Bulb area to cortical and cortical plus IRS diameter

Table 3.4 **Relationships of cortical (DC) and cortical plus IRS (DI) diameter to three different bulb area measurements**

	JUNE		AUGUST		NOVEMBER	
	DI	DC	DI	DC	DI	DC
AW	0.436	0.452	0.576	0.531	0.141	0.467
AP	0.528	0.438	0.512	0.519	0.094	0.430
AS	0.413	0.430	0.616	0.630	0.144	0.457

The relationships of DC and DI with areas is not as strong as with diameters (Table 3.1). But both DI and DC are very similar in the degree of relationship with areas, except for DI being only weakly related to areas in summer (Table 3.4).

Dermal papilla dimensions

Table 3.5 gives the correlation coefficients for dermal papilla length (PL) and area (PA) to diameters and areas. The highest consistent relationship was seen in AP to PL (r=0.9) and AP to PA (r=0.75). A slightly weaker relationship existed between AS and PL (r=0.6) and AS and PA (r=0.6). In summer PA (r=0.6) was more highly correlated with the different diameters than PL (r=0.44). PA and PL showed a relationship of r=0.69 (Table 3.6).

Table 3.5 **Correlation coefficients of dermal papilla length (PL) and dermal papilla area (PA) to bulb diameter and bulb area measurements.** Parameters which are consistently closely linked at all three stages of the year are shown in grey filled cells.

	PL			PA		
	JUNE	AUGUST	NOVEMBER	JUNE	AUGUST	NOVEMBER
DW	0.437	0.500	0.448	0.454	0.554	0.604
DP	0.422	0.474	0.456	0.420	0.525	0.607
DS	0.430	0.789	0.424	0.452	0.537	0.581
DF	0.422	0.472	0.458	0.420	0.524	0.607
AW	0.533	0.554	0.517	0.353	0.406	0.567
AP	0.907	0.919	0.930	0.755	0.727	0.807
AS	0.564	0.609	0.623	0.530	0.625	0.601
AC	0.65	0.54	0.52	0.61	0.47	0.40

Table 3.6 **Correlation coefficients for relationships of PL to PA**

	JUNE	AUGUST	NOVEMBER
	PL	**PL**	**PL**
PA	0.714	0.644	0.728

Number of labelled cells in the follicle bulb

Table 3.7 **Pooled within-class correlation coefficients for relationship of the number of labelled cells (NO) to all other variables for June, August and November.** Closely correlated parameters are shown in grey filled cells.

	JUNE	AUGUST	NOVEMBER
	NO	NO	NO
DW	0.57	0.46	0.38
DP	0.56	0.44	0.30
DS	0.57	0.45	0.40
DF	0.56	0.44	0.30
PL	0.29	0.35	0.24
PA	0.01	-0.01	-0.03
DI	0.33	0.38	0.09
DC	0.17	0.26	0.18
AW	0.43	0.40	0.31
AP	0.42	0.42	0.28
AS	0.65	0.62	0.51
AC	0.47	0.34	0.51

Correlation Coefficients

The closest consistent relationship to the number of labelled/proliferating cells in the bulb (NO) throughout the entire observation period was to the bulb area (AS) with r=0.6, which was only slightly influenced by a change in season. The area AC was less closely related to NO. Diameter measurements (DW, DP, DS, DF) were equally highly correlated (r=0.5) but only in June. Dermal papilla length had a relationship of r=0.3 throughout the entire observation period with only a small drop in summer. Papilla area, however, showed almost none to even a slight negative relationship. Seasonal variations in the degree of relationship to the number of labelled cells were seen in the bulb diameter (DP, DF) as well as bulb area (AP) measurements associated with the height of the papilla. Both showed a drop in their relationship with NO during summer. Furthermore, the diameter of cortex plus IRS (DI) at their origin, although never expressing a strong relationship to NO, declined during summer (Table 3.7).

Total number of cells in bulb area

Because phenotypic extremes had been selected deliberately, the subset of samples chosen for FWT and CLT sheep was significantly different in the mean number of labelled cells. The difference in the mean number of unlabelled cells only just reached significance in November. Follicles of FWT sheep had more cells in the bulb area (AP) than follicles of CLT sheep, from which more cells were labelled than unlabelled. FWT follicles had 54% labelled cells in June, 56% in August and 58% in November in skin samples taken 2 hours after i.c. BrdU injection (see Chapter 2, page: 2.4). In CLT sheep follicles, labelled cells never reached the 50% mark. All these differences were significant (Table 3.8).

Table 3.8 **Significance levels for analysis of variance of differences in mean values of cell numbers in the follicle bulb area.** Results for FWT follicles are given in grey shaded cells.

	JUNE		AUGUST		NOVEMBER	
N	40		39		40	
UNLABELLED CELLS						
FWT	46.4	±2.72	38.6	±2.57	40.9	±2.39
CLT	39.3	±1.99	44.7	±3.75	45.0	±3.40
F	1.00		1.75		4.44	
p	0.324	NS	0.19	NS	0.042	*
LABELLED CELLS						
FWT	54.1	±2.58	48.05	±2.09	57.9	±2.48
CLT	31.4	±2.62	29.2	±1.39	36.3	±1.60
F	37.99		57.46		53.30	
p	0.0001	***	0.0001	***	0.0001	***
TOTAL NUMBER OF CELLS						
FWT	100.7	±4.00	86.6	±3.51	98.7	±3.83
CLT	70.7	±4.19	73.9	±4.50	81.3	±4.22
F	26.49		4.94		9.33	
p	0.0001	***	0.032	*	0.004	***

	JUNE		AUGUST		NOVEMBER	
N	40		39		40	
RATIO OF LABELLED/UNLABELLED CELLS						
FWT	1.253	±0.099	1.329	±0.089	1.499	±0.103
CLT	0.798	±0.049	0.734	±0.0614	0.878	±0.072
F	16.9		30.73		24.65	
p	0.0001	***	0.0001	***	0.0001	***
PROPORTION OF LABELLED/TOTAL CELLS						
FWT	0.541	±0.019	0.559	±0.018	0.588	±0.015
CLT	0.436	±0.016	0.410	±0.019	0.455	±0.018
F	18.67		31.83		32.31	
p	0.0001	***	0.0001	***	0.0001	***

Relationship of the total number of cells to the number of proliferating cells

Regression analysis revealed no significant relationship between labelled and unlabelled cells, but there was a relationship between labelled cells and the total number of cells in the bulb area. This relationship was relatively constant in follicles of FWT sheep in June, August and November, whereas in follicles of CLT sheep, seasonal variations became apparent. In June, the number of labelled cells was closely related to the total number of cells. A weaker relationship was seen in August, whereas in November it was intermediate (Table 3.9).

Table 3.9 **Relationship of mean number of labelled cells to total number of cells (R-sq) and significance levels for analysis of variance of these relationships for FWT and CLT follicles.** Results for FWT follicles are given in grey shaded cells

	JUNE		AUGUST		NOVEMBER	
N	40		39		40	
FWT	R-sq=54.4%		R-sq=47.5%		R-sq=42.1%	
F	21.54		15.35		13.07	
p	0.0001	***	0.001	***	0.002	***
CLT	R-sq=86.9%		R-sq=42.6%		R-sq=63.3%	
F	119.23		13.35		31.06	
p	0.0001	***	0.002	***	0.0001	***

Relationship of the total cell number to follicle bulb and dermal papilla dimensions

In follicles of CLT sheep, a stronger overall relationship of the number of cells to bulb area was apparent, which was strongest in winter (Table 3.10).

Both area measurements excluded the dermal papilla in order to avoid the possible influence of dermal papilla changes.

In follicles of FWT sheep, a weaker relationship to follicle dimensions was apparent, which for two of the measurements did not reach significance.

Table 3.10 **Significance levels for analysis of variance on the relationship (R-sq) of the total cell number to follicle bulb and dermal papilla dimensions.** Results for FWT follicles are given in grey shaded cells

	JUNE		AUGUST		NOVEMBER	
N	40		39		40	
	FWT	CLT	FWT	CLT	FWT	CLT
	corrected AP (AP-PA)					
R-sq	55.1%	80.1%	38.1%	76.2%	45.1%	53.1%
F	22.12	72.58	10.45	57.75	15.09	20.36
p	0.0001	0.0001	0.005	0.0001	0.001	0.0001
	corrected AS (AS-PA)					
R-sq	21.5%	85.1%	46.3%	59.7%	35.3%	66.3%
F	0.47	103.0	14.64	26.68	9.82	35.34
p	NS	0.0001	0.001	0.0001	0.006	0.0001
	papilla length (PL)					
R-sq	34.1%	50.8%	18.3%	59.2%	48.0%	41.6%
F	9.32	18.57	3.8	26.15	16.63	12.8
p	0.007	0.0001	NS	0.0001	0.001	0.002
	bulb diameter (DP)					
R-sq	44.2%	50.5%	22.8%	63.9%	22.9%	36.2%
F	14.25	18.38	5.03	31.84	5.33	10.22
p	0.001	0.0001	0.039	0.0001	0.033	0.005

Derived variables

Table 3.11 **Least square means of general linear model analysis of variance and significance levels for group x month effect of variable NO/AS.**

Number of observations: 42

	FWT		AOV	CLT	
	LSMEAN	SEM	p	LSMEAN	SEM
JUNE	0.0056	0.0002	0.1152	0.0067	0.0002
AUGUST	0.0067	0.0002	0.0236	0.0056	0.0002
NOVEMBER	0.0045	0.0002	0.2821	0.0042	0.0002

Table 3.12 **Least square means of general linear model analysis of variance and significance levels for group x month effect of variable NO/AP**

Number of observation: 42

	FWT		AOV	CLT	
	LSMEAN	SEM	p	LSMEAN	SEM
JUNE	0.0052	0.0002	0.5324	0.0055	0.0003
AUGUST	0.0063	0.00015	0.9230	0.0064	0.0003
NOVEMBER	0.0042	0.00016	0.8762	0.0042	0.0003

Table 3.13 **General linear model analysis of variance significance levels for NO/AS and NO/AP on group x month effect in FWT and CLT sheep**

NO/AP		JUNE	AUGUST	NOVEMBER
		NO/AS		
JUNE	FWT		0.0006	0.0001
	CLT		0.717	0.0001
AUGUST	FWT	0.0168		0.0016
	CLT	0.0976		0.0001
NOVEMBER	FWT	0.0001	0.0098	
	CLT	0.0084	0.0001	

Table 3.14 **Least square means of general linear model analysis of variance and significance levels for group effect of variable NO/DS.** Number of observations: 930

	FWT		AOV	CLT	
	LSMEAN	SEM	p	LSMEAN	SEM
JUNE	0.3046	0.0062	0.0307	0.2859	0.006
AUGUST	0.3528	0.00053	0.0001	0.3011	0.0062
NOVEMBER	0.3037	0.0053	0.0181	0.2859	0.0053

Table 3.15 **Least square means of general linear model analysis of variance and significance levels for group effect of variable NO/DP.** Number of observations: 928

	FWT		AOV	CLT	
	LSMEAN	SEM	p	LSMEAN	SEM
JUNE	0.3068	0.0064	0.0181	0.286	0.0061
AUGUST	0.3537	0.0054	0.0001	0.3018	0.0063
NOVEMBER	0.3031	0.0055	0.0478	0.2879	0.0054

Table 3.16 **General linear model analysis of variance significance levels for NO/DP and NO/DS on month effect in FWT and CLT sheep**

NO/DS		JUNE	AUGUST	NOVEMBER
		NO/DP		
JUNE	FWT	-	0.0001	0.6565
	CLT		0.0722	0.822
AUGUST	FWT	0.0001	-	0.0001
	CLT	0.0775		0.0947
NOVEMBER	FWT	0.912	0.0001	-
	CLT	0.9934	0.7526	

Table 3.14 **Least square means of general linear model analysis of variance and significance levels for group x month effect of variable NO/DS.**

Number of observations: 42

	FWT		AOV	CLT	
	LSMEAN	SEM	p	LSMEAN	SEM
JUNE	0.3046	0.0062	0.4783	0.2859	0.015
AUGUST	0.3528	0.0053	0.0188	0.3050	0.015
NOVEMBER	0.3037	0.0053	0.6117	0.2869	0.015

Table 3.15 **Least square means of general linear model analysis of variance and significance levels for group x month effect of variable NO/DP.**

Number of observations: 42

	FWT		AOV	CLT	
	LSMEAN	SEM	p	LSMEAN	SEM
JUNE	0.2916	0.015	0.4313	0.291	0.0129
AUGUST	0.3057	0.015	0.0190	0.356	0.0129
NOVEMBER	0.2902	0.015	0.7583	0.296	0.0129

Table 3.16 **General linear model analysis of variance significance levels for NO/DP and NO/DS on group x month effect in FWT and CLT sheep**

		JUNE	AUGUST	NOVEMBER
			NO/DP	
JUNE	FWT		0.0149	0.5533
	CLT		0.5112	0.4725
AUGUST	FWT	0.0001		0.0036
	CLT	0.5117		0.9502
NOVEMBER	FWT	0.9120	0.0001	
	CLT	0.8417	0.3939	

Table 3.17 **Least square means of general linear model analysis of variance and significance levels for group x month effect of variable NO/PL.**

Number of observations: 42

	FWT		AOV	CLT	
	LSMEAN	SEM	p	LSMEAN	SEM
JUNE	0.457	0.021	0.803	0.448	0.0246
AUGUST	0.503	0.021	0.0503	0.4679	0.0246
NOVEMBER	0.416	0.021	0.557	0.3961	0.0246

Table 3.18 **General linear model analysis of variance significance levels for NO/PL on group x month effect in FWT and CLT sheep**

		JUNE	AUGUST	NOVEMBER
		NO/PL		
JUNE	FWT		0.1404	0.1856
	CLT		0.5811	0.1463
AUGUST	FWT			0.0081
	CLT			0.0503
NOVEMBER	FWT			
	CLT			

Area density ratios

Relating the number of proliferating cells to two different areas of germinative zone (NO/AP and NO/AS) no difference between lines are apparent, but significant monthly changes in the ratio with the highest ratio in August and the lowest in November (Tables 3.11, 3.12 and 3.13).

The density ratio NO/AS shows no significant monthly changes for CLT sheep between June and August (Table and 3.13).

Diameter ratios

Both diameter ratios show similar results, with line differences in June and November but none in August, and without seasonal variations in CLT sheep (Tables 3.14, 3.15 and 3.16).

Papilla ratios

Number of proliferating cells related to papilla length yields no line differences. In August the line difference shows a trend toward significance. A month effect can be seen between August and November in both FWT and CLT sheep (Tables 3.17 and 3.18).

DISCUSSION

Bulb diameters

Bulb diameter measurements were highly correlated. It should thus be sufficient to measure only one diameter.

DC and DI were related in winter but not in summer. In summer, cortical plus IRS diameter was unrelated to other bulb diameters (r=0.19). Since bulb cells form both cortex and IRS, and bulb diameter and cortical diameter retain a strong relationship (Table 3.1) little influenced by season, a relative independence and irregularity in IRS width is suggested. Hence, diameter variations in the IRS (Chapter 4, page: 4.18) occur and are not necessarily a reflection of follicle size or cortical diameter. Especially in periods of high productivity (summer), bulb diameter reflects cortical diameter, and the IRS plays a negligible role in dimensional relationships. This indicates that at bulb level, a redistribution of cells (proportions/numbers) to either structure must take place (Priestley, 1967a), which depends on seasonal modulations. It seems that the follicle exerts a certain influence on the upward flowing cells lines to redirect these cells into either cortex or IRS depending on seasonal and/or genetic influences. Consequently, one needs to be careful in relating bulbar proliferation rates or dimension directly to fibre output. The importance of investigating the proportions of cells forming IRS and cortex becomes apparent.

Follicle bulb areas

Bulb areas (uncorrected for dermal papilla area) were consistently related over the three months, and were therefore not seasonally influenced in their relationship. The close relationship of AP and AS suggests an influence of the dermal papilla on cellular events in the follicle bulb matrix, since by definition, the size of the area AP is strongly influenced by the size of the dermal papilla area, and AS by the spatial distribution of labelled cells. That is, if AP and AS are positively related, it indicates that the spatial distribution of proliferating cells within the bulb matrix could be closely associated with dermal papilla dimensions. Bulbar dimensions and papilla dimensions are also linked (Table 3.5). The associations of bulb areas to DI and DC were weaker than to bulb diameters (Table 3.4), although they followed a similar seasonal

pattern; strongest in August, and weakest in November. In conclusion, it is suggested that bulb diameters and cortex diameters are related, and one is best predicted by the other one, rather than predicting cortex diameter by follicle bulb area.

Dermal papilla

That the dermal papilla and the follicle bulb parameters are closely correlated suggests a close functional interrelationship. If measured areas represent the germinative population, then this result in sheep follicles supports the suggestion by VanScott et al. (1963) in hair follicles of humans that a close relationship exists between the number of germinative cells and the size of the dermal papilla. Seasonal variations in the relationship of bulb diameters to papilla area were more pronounced than those to papilla length, indicating that the papilla area might have a greater influence on bulb width.

Of the bulb areas, AP is related most strongly to papilla dimensions, since by definition, the uppermost boundary of this area is determined by the height of the papilla. Nevertheless, a reasonably strong relationship exists to AS which could be an indication of a dependence on the spatial distribution of the proliferating cells and the arrangement of germinative tissue to dermal papilla dimensions. The weaker relationship to AW and AC demonstrate the necessity for the bulb area measurements to encompass the germinative zone. The strong relationship of papilla area and papilla length to AS and AP is further evidence that these areas might best represent the germinative region. This confirms the observations of VanScott et al. (1963) that the volume of the fibre matrix is proportional to the height of the papilla, and that a close relationship exists between the population of germinative cells and the size of the hair papilla. Papilla size (i.e. length) limits the size of the germinative population producing the hair or wool fibre.

Number of proliferating cells

In general, the number of proliferating cells (NO) appeared to be more closely related to bulb diameters than to bulb areas at each stage of the year. The area AS can be expected to be related to NO since, by definition, its distal boundary is related to the spatial distribution of labelled cells, and hence the extent of the germinative tissue. Closer relationships seem to occur during periods of low production. A surprising result was the weak relationship between NO and the area AC. It was assumed that the definition of the boundary of AC (Short et al., 1965) would be the most precise to represent the area of germinative tissue. Only a weak association

with the dermal papilla variables was found, suggesting that papilla dimensions, although being related to bulb areas, have little influence on the number of proliferating cells.

A possible conclusion is that the control of growth of the fibre works through the papilla to influence the germinative zone of the bulb which, in turn, is able to grow, thereby including more cells in its germinative zone. If this were to result in more proliferating cells, fibre growth is increased. This concept is expanded further in the general Discussion (page: D.5).

Total number of cells in the follicle bulb

Phenotypic extremes of FWT sheep had a higher percentage of proliferating cells in the germinative cell population, as well as a larger total cell population, which occupied a larger bulb area. In both flocks, the number of proliferating cells was related to the total number of cells in the population, this relationship being modulated seasonally, especially in follicles of CLT sheep (Table 3.9). In follicles of CLT sheep, a closer relationship of labelled to total cells was apparent. Follicles of CLT sheep consistently had a lower percentage of labelled cells than FWT follicles, hence the advantage of FWT sheep appears independent of the total cell number. Thus in FWT follicles, cell proliferation rate may be an additional factor influencing cell production (see Chapter 4, page: 4.26).

The total number of cells forming the germinative tissue zone in the follicle bulb was subject to seasonal changes, and related to bulb size; especially in the CLT follicles. Although as a general principle of tissue growth, an increase in the size of cells or in intercellular substance may also affect tissue growth (Baserga, 1989), this plays only a minor role in follicle bulb growth. The germinative cells in the follicle bulb can be regarded as vegetative intermitotic cells, which continually divide in the adult organism, thus serving as a cell reservoir (Bertalanffy, 1960). The activity of these cells is concerned with mitosis (proliferative cell compartment) while in the upper layers they gradually undergo keratinization (differentiating cell compartment). This includes the synthesis of cytoplasmic protein (Wright, 1983) associated with cellular hypertrophy. The only cell growth occurring in germinative tissue is that of postmitotic cells having to double their size before entering their next mitosis (Baserga, 1984).

Within a selection line, the number of proliferating cells in the bulb is a constant proportion of the total number of cells in the population. However, the proportion of cells proliferating varies between the selection lines (or with different levels of fibre production). Therefore, a high production capacity is the result of both a larger follicle size and therefore a greater germinative cell population, and also a higher proportion of proliferating cells within

that population.

Density ratios

Because the boundary line for AS can intersect the dermal papilla (Figure 3.2), the corrected area AS might not always be a valid measurement. Seasonal monthly changes in the ratios (NO/AP and NO/AS), are mainly a consequence of area changes rather than changes in NO. Although both variables show a winter decline and a summer increase, these modulations were not strictly proportional, visible in the decrease of the ratios. Similarities in these ratios between the two lines of sheep indicate that they have similar densities of mitotic cells in their follicle bulbs.

Nevertheless, other variables, when related to NO (NO/DS and NO/DP), show a potential for line differences in mitotic density, suggesting that changes in the density of cells might be part of between line variations. This contradiction cannot be resolved from the current study. Possible explanations for differences in mitotic density could be either additional fluctuations in cell proliferation rates (Chapter 4, page: 4.39) or volume changes in cell size. These were not investigated in this study, but should be given attention in future investigations.

Chapter 4
A Comparative Seasonal Study of Wool Follicle Morphology and Cell Proliferation in New Zealand Romney Sheep Selected for High Fleece Production

INTRODUCTION

Cellular events associated with wool production

Because the follicle bulb provides all the cells forming the fibre, the IRS and the ORS, wool production must be influenced by cellular events in the follicle bulb. The total output of the follicle is a direct function of the size of the germinative region of the bulb and the proliferative activity of the cell population of this region of the bulb. Migration of newly formed cells, cell loss and changes in the morphology and distribution of cells into various follicular structures determines the proportional net output of fibre.

Factors altering wool production, measured as volumetric output of fibre material, may be genetic as well as nutritional or seasonal. Response to these influences could be brought about by changes in the follicle population, follicular dimensions and cell producing events in the bulb.

Nutritional effects on wool production in the Merino

Short et al. (1965) examined the relationship between wool production and the mitotic rate of bulb matrix tissue of only one Merino sheep at two subsequent levels of nutrition using intravenous injection of colchicine. Some of the variables measured were the frequency of inactive follicles, which decreased from 2.1% to 0.7% with a change to a higher nutritional level; and fibre length and diameter, which both also responded to nutritional changes. The number of mitoses in the bulb matrix as well as the germinative cell population increased with the higher nutritional plane. Bulb width appeared to be affected by nutrition. Wilson and Short (1979), who studied Merino sheep under varying nutritional conditions, also concluded that most variation in fibre output was caused by the number of germinal cells in the bulb and their proliferation rate (r=0.95). Schinckel (1962) studied fibre and bulb diameter changes in Merinos

caused by dietary intake, as well as mitotic rate. Because cell number increase did not account solely for higher fibre output, he suggested that there might be an increase in fibre cortical cell size. This suggestion could only partly be supported by Williams and Winston (1987). Hynd (1989a) studied the effects of nutrition on the follicles of sheep breeds (Merino and Corriedale) genetically different in wool production. Fibre length growth rates and diameter changes responded to nutrition, with fibre length growth increasing to a greater extent. Hynd and Everett (1990) compared the effects of high and low levels of nutrition on strongwool Merino sheep, measuring cell birth rate (CBR) in the follicle bulb. He concluded that CBR determines the rate of fibre growth. CBR was dependent on nutrition and closely related to fibre growth. Hynd used either colchicine or BrdU to label proliferating cells, both methods giving similar results although colchicine repeatedly gave lower absolute values. He concluded that colchicine appeared to depress CBR and that previous numbers of proliferating cells had been underestimated. In Hynd's (1989a) study, the level of nutrition affected the germinative tissue volume and the number of bulb cells proliferating per unit time.

Selection for increased wool production in the Merino

Investigations of the effects of selection for increased wool production on follicle morphology have been scarce. Williams and Winston (1987) measured follicle and fibre characteristics in Merino sheep selected for genetic differences in wool production ("Fleece Plus" versus "Fleece Minus") under different feeding regimes. "Fleece Plus" sheep, in general, exhibited higher follicle density and greater wool production per unit area of skin. Higher nutritional levels affected "Fleece Plus" sheep more than "Fleece Minus" sheep, these effects being detectable as increases of fibre length and diameter in "Fleece Plus" sheep. Bulb diameter was neither under genetic nor under nutritional influence, but "Fleece Plus" sheep had a greater area of mitotically active tissue, which was independent of nutritional levels. Using colchicine injected i.c. to arrest proliferating cells in metaphase, no genetic or nutritional differences could be found in increase of the number of arrested cells. A recent study by Hocking Edwards and Hynd (1992), comparing strongwool and finewool Merinos, showed that the genotype determined the difference in the volume of the mitotically active tissue. Although strongwool Merinos also showed a higher bulb cell production rate, wool production was best predicted by the volume of germinative tissue and the follicle density.

Distribution of bulb matrix cells to cortex and IRS

Only recently has the possible influence of cell distribution to different follicular structures, especially the IRS and fibre, been considered as a determinant of fibre production. For the Merino sheep, Short et al. (1965) claimed that only a small proportion of dividing matrix cells finally enter the fibre as compared with the proportion migrating to form the IRS. Their observations make fibre production appear an inefficient process. The proportional distribution of cells to either fibre or IRS was not considered to be related to nutritional effects but rather to genetic differences between Merino sheep (Wilson and Short, 1979). Hynd (1989a) suggested a strong genetic component to cell distribution using the ratio of cortical cells per hour to bulb cell mitotic density as an indicator for the distribution to the cortex, derived from data of Williams and Winston (1987). These ratios were higher in sheep genetically superior in wool production, indicating that more bulb cells migrated into the fibre cortex. Thus, genetic selection for higher wool production appears to influence the proportional distribution of cells to either of the abovementioned structures (Hynd, 1989a; Hocking Edwards and Hynd, 1992).

However, it needs to be established whether fibre and IRS cells move upwards together and at the same speed or if certain cell lines show an accelerated migration. Although IRS cells are believed to move up the bulb ahead of contemporaneously formed cortical cells (Epstein and Maibach, 1969; Chapman, 1971; Chapman et al., 1980) they still have to move at a similar rate in order for cuticular scale patterns of the wool fibre to form (Woods and Orwin, 1982). The possibility of the existence of different velocities between cell lines within either IRS or cortex forming cells appears to be highly unlikely. Thus, the IRS can only accommodate a high cell number without a significant size increase, if cells either decrease in size or die. If, as calculated by Short et al. (1965) only a proportion of 20% of the bulb matrix cells appear in the fibre, and assuming no cell death, the majority of cells might migrate to form the IRS. This assumption must be questioned and it has yet to be investigated whether genetic selection for superiority in wool production influences the distribution of matrix cells.

Factors influencing the follicle bulb cell population and fibre output of the Romney sheep

Several sheep breeds, including the Romney, were studied by Fraser (1965), who claimed that a reduction in mitotic density occurred parallel to an increase in germinative tissue volume within the follicle population of Romney sheep. The increase in the latter was caused largely by an increase in cell number. This change in volume may not necessarily have a causal

connection to changes in cell turnover time. Furthermore, he did not directly relate fibre length or diameter changes to the same control mechanisms such as cellular events in the bulb. Henderson (1965) emphasised the significance of germinative tissue volume in seasonal as well as nutritional changes in Romney sheep. Other parameters, influencing fibre output, in terms of changes in the follicle population, are discussed in Chapter 1 (page: 1.1).

In conclusion, previous studies have shown that dimensional changes in the wool follicle do influence fibre output. The size of the follicle bulb is a reflection of the number of cells rather than the size of cells encompassed within it. The total number of cells in the germinative tissue region contributes to the number of mitotically active, proliferative cells. Whether the change of proliferating cell number is merely a reflection of size changes of the germinative tissue, rather than being due to a rate increase, has yet to be determined. Studies have seldom used Romney sheep and even the information available for Merino sheep is equivocal. Furthermore it is not clear if results obtained from studies on Merinos can be adapted to the Romney. Few studies have tried to relate measurements on fibre output (fibre length, fibre diameter) directly to measurements undertaken on the morphology of the underlying follicle population. No study has related the components of fibre growth and follicle morphology in Romney sheep that have been genetically selected for different levels of wool production.

The experiment described here was conducted to examine the relative contribution of various follicle parameters to fibre output in sheep differing genetically in clean wool production per day during periods of low (winter) and high (summer) wool production. Fibre output was determined as daily fibre length growth rate, fibre diameter, follicle density and percentage of active follicles in the population. Individual follicular influence on wool production was assessed by the measurement of several dimensional variables of the follicle bulb, including the dermal papilla, and by estimating the proliferative activity of bulb cells.

MATERIALS AND METHODS

Animals

Twenty New Zealand Romney rams from each of two flocks, selectively bred for either high fleeceweight (FWT) or at random (CLT) were taken for this experiment. The rams were kept outside, feeding on the same pasture. Details of the selection lines are given in the general Introduction (page: I.26).

Fibre length growth measurements

In order to minimize errors due to an inaccurate method of measuring fibre length growth between dye-bands (Chapter 1, page: 1.19), a better method was developed which allowed identification and measurement of individual fibres. Dye-banded fibre samples were taken for measurement of fibre length both in winter (growth period from June until August, 57 days) and insummer(growth period from November until December, 34 days). A description of the method for dye-banding is given in the general Materials and Methods (page: M.2). The butt of the fibre was identified by the absence of dye, whereas the distal end always showed a band. Each fibre sample was trimmed with scissors from the tip down to the edge of the distal band, before mounting individual fibres on tape. In order to inspect the dye-bands of individual fibres, separate fibres from each sample were attached to strands of double sided tape, which was then covered with acetate foil on both sides. Fibres were selected for mounting in a manner that ensured that they originated randomly from several different wool staples. By holding the foil over a white background, fibre length was measured using a digitizing pad (Sigma-Scan, 3.9, Jandel Scientific, Corte Madera, CA, USA) followed by analysis with the appropriate software (Minitab 7.2, Minitab. Inc.). In contrast to samples for other measurements, taken twice in winter, fibre samples were only taken once during winter.

Fibre diameter measurements

A subsample of a clipped dye-band fibre sample was analyzed for fibre diameter in June, September and December at the Whatawhata Fibre Testing Centre. Measurements were taken from snippets cut close to the fibre butt using a CSIRO Fibre-Fineness Distribution Analyzer (Lynch and Michie, 1973). This analyzer uses an optical interference method to measure the distribution of fibre diameters in a fibre sample. It works on the principle of recording the magnitude of a shadow cast by the fibre on a light-sensitive cell as the fibre passes through a laser beam. Fibre snippets are manually fed into a mixing bowl where they are suspended in isopropanol. The liquid carries the snippets to a glass measurement cell. The cast shadow of the passing fibre produces a pulse, the magnitude of which is calibrated to a fibre diameter measurement and used for the storage of diameter distribution of the sample (Irvine and Lunney, 1979). A total of 45 fibre samples were analyzed, with 100 snippets per sample. (June: 8 FWT and 9 CLT; September: 6 FWT and 8 CLT; December: 6 FWT and 8 CLT).

Fibre volume output

Fibre volume output was calculated from fibre length growth and fibre diameter as already described in Chapter 1 (page: 1.7).

Secondary to primary follicle ratio

Efficiency of wool production of the follicle group was assessed on cross-sectional sacpic slides by estimation of the ratio of secondary to primary follicles (S:P ratio). Details of the staining protocol are given in the Appendix (page: A.4) and in the general Materials and Methods (page: M.4). In order to differentiate secondary from primary follicles, in sections cut at mid-sebaceous gland level, 10 trio groups were counted per section for each sheep in June, August and November respectively. Only active follicles were considered in the count (Table 4.7). Identification of primary and secondary follicles followed the criteria described in Chapter 1 (page: 1.7) and in the general Materials and Methods (page: M.4). Some data for S:P (and percentage of anagen follicles) for August were missing, due to problems in the histological preparation of the samples.

Activity of follicle population

For the evaluation of overall follicle population activity, serial sections stained with sacpic were screened under the light microscope. During catagen, the staining properties of the IRS, as it gradually degenerates, change from bright red to orange. This colour change is occasionally associated with a change in shape from a smooth circle to a more irregular shape. Cell nuclei of the ORS change in shape from round, with an orientation following the circular structure of the ORS in cross-section, to ovoid, orientated radially to the follicle axis. The connective tissue sheath may be larger in some catagen follicles compared to their size in anagen (Ryder and Stephenson, 1968).

Only a distinction between anagen and catagen was observed. Other stages (telogen), as described in the general Introduction (page: I.26), were not seen. In describing activity levels of follicles the term "inactive" was used for every stage other than anagen. The occurrence of catagen follicles was recorded for a total of 100 follicles, at each of 5 different skin levels. Samples were taken for estimation of active follicles from a total of 19 sheep, twice during the winter and once during summer. Figure A.1 (page: A.5) in the Appendix illustrates the morphological changes in the follicle during catagen, as observed in cross-section. The photomicrographs were taken as examples from serial cross-sections.

Follicle bulb measurements

Measurements undertaken on individual bulbs included:

a) number of labelled, proliferating cells in the proliferative region of the bulb **(NO)**

b) bulb diameter **(DS)**

c) bulb area (including papilla) **(AP)**

d) papilla area **(PA)**

e) papilla length **(PL)**

f) cortex diameter at the origin of the fibre **(DC)**

g) cortex and IRS diameter at the origin of the fibre **(DI)**

Animals received an i.c. injection of BrdU (0.1ml of 3mg/ml BrdU solution) into their clipped midside skin as described in Chapter 2 (page: 2.4). Skin biopsies were taken from FWT and CLT sheep in June, August and November, and prepared for wax embedding as described in the general Materials and Methods (page: M.3). Histological tissue sections were subject to the immunocytochemical detection of proliferating bulb matrix cells described in Chapter 1 (page: 1.10). About 20 representative bulbs in longitudinal skin sections were chosen from each animal. The criteria of a 'representative' bulb are given in Chapter 2 (page: 2.7). A light microscope with a video camera attached was connected to a PC-computer. The captured histological image on the computer screen was measured using specially developed computer software (MARKLINE, Dr. A.J. Hall, Fruit and Trees, DSIR, Palmerston North).

RESULTS

Fibre length measurements

Table 4.1 **Mean daily fibre length growth rates (mm d^{-1}) of control (CLT) and fleeceweight (FWT) sheep during summer (November, December) and winter (June - August)**

SEASON	GROUP	N	MEAN	SEM
WINTER	CLT	7	0.562	0.058
	FWT	9	0.572	0.051
SUMMER	CLT	8	0.683	0.061
	FWT	5	0.881	0.096

Table 4.2 **Level of significance for analysis of variance for daily fibre length growth rates**

[A] Between groups. within seasons

SEASON	F	p
WINTER	0.03	0.861
SUMMER	3.39	0.093

[B] Within groups, between seasons

GROUP	F	p
CLT	2.03	0.178
FWT	6.98	0.03

During winter, FWT showed only a slight advantage over CLT sheep in length growth rates. This trend was not significant in either season.

FWT sheep showed a significant seasonal change in fibre length growth rate, in contrast to CLT sheep.

Fibre diameter measurements

Table 4.3 **Mean values for fibre diameter (µm) of control (CLT) and fleece-weight (FWT) sheep at three stages of the year**

MONTH	GROUP	N	MEAN	SEM
JUNE	CLT	8	36.5	1.49
	FWT	7	36.61	0.89
SEPTEMBER	CLT	8	31.45	1.66
	FWT	6	35.48	2.17
DECEMBER	CLT	8	40.81	1.028
	FWT	6	43.82	0.789

Table 4.4 **Significance levels for analysis of variance F-values and significance levels of fibre diameter measurements**

[A] between groups, within seasons		
MONTH	F	p
JUNE	0.00	0.95
SEPTEMBER	2.27	0.158
DECEMBER	4.77	0.05

[B] within groups, between seasons		
GROUP	F	p
CLT	10.94	0.001
FWT	10.29	0.001

The frequency distribution for fibre diameter of all samples was normal. In June, mean values for fibre diameter of CLT and FWT sheep were similar. In September and December, fibres of FWT sheep had a larger diameter than CLT sheep. In both groups, the narrowest fibres occurred during the late winter-early spring period (September). No significant difference could be found in fibre diameters of control (CLT) and fleeceweight (FWT) sheep during the winter (June) or early spring (September). In summer the difference was significant, with the fleeceweight sheep producing coarser wool than the control sheep. Both CLT and FWT showed a marked seasonal change in fibre diameter, the fibre diameter being smaller in winter than in summer. The seasonal variation was more pronounced in the control sheep.

Fibre volume output

Table 4.5 **Mean values for fibre volume output (mm^3 d^{-1}) for FWT and CLT sheep and significance levels for analysis of variance for group differences at three stages of the year**

		JUNE	SEPTEMBER	DECEMBER
N		14	12	10
FWT	MEAN	$6.3x10^{-3}$	$5.8x10^{-3}$	$12.0x10^{-3}$
	SEM	$0.7x10^{-3}$	$0.8x10^{-3}$	$1.4x10^{-3}$
CLT	MEAN	$6.2x10^{-3}$	$4.4x10^{-3}$	$8.3x10^{-3}$
	SEM	$1.1x10^{-3}$	$1.1x10^{-3}$	$1.0x10^{-3}$
F		0.00	1.11	4.62
p		0.98	0.32	0.05

Table 4.6 **Significance levels for analysis of variance of month effect within groups**

GROUP	F	p
FWT	11.38	0.001
CLT	3.29	0.063

At all three stages of the year, FWT sheep showed a higher fibre volume output than CLT sheep, but the difference was only just significant in summer (Table 4.5). Seasonal changes in fibre output were only significant in FWT sheep (Table 4.6), but CLT sheep showed a significant effect between September and December (F=6.94/ p=0.025). Advantages of FWT over CLT sheep in fibre output was only 1.6% in June, but 31.8% in September and 49.6% in December.

Secondary to primary follicle ratio (S:P ratio)

Table 4.7 **Mean values of S:P ratio for individual sheep at three stages of the year**

(*: denotes missing values, page: 4.6)

	IDENTIFICATION NUMBER	JUNE	AUGUST	NOVEMBER
FWT	26	7.13	5.65	6.25
	28	5.41	*	5.81
	37	*	5.62	5.83
	39	6.06	4.91	5.67
	42	4.83	4.44	6.77
	72	5.77	5.58	6.26
	73	6.25	*	5.75
	120	6.67	*	6.97
	199	5.71	*	6.36
CLT	51	5.96	5.03	5.80
	147	5.37	5.36	5.86
	159	5.68	5.86	5.17
	182	4.49	*	*
	185	5.19	5.60	5.51
	186	5.66	5.50	5.65
	190	5.69	*	5.53
	203	6.00	*	6.23
	204	5.70	4.88	5.83
	247	*	*	5.67

Table 4.8 **Mean values for S:P ratios of FWT and CLT sheep at three stages of the year**

MONTH	GROUP	N	MEAN	SEM
JUNE	CLT	9	5.53	0.15
	FWT	8	5.98	0.25
AUGUST	CLT	6	5.37	0.15
	FWT	5	5.24	0.24
NOVEMBER	CLT	9	5.69	0.10
	FWT	9	6.19	0.15

Table 4.9 **Significance levels for analysis of variance of the S:P ratio of group means**

[A] between groups, within seasons		
MONTH	F	p
JUNE	2.42	0.141
AUGUST	0.23	0.642
NOVEMBER	7.23	0.016

[B] within groups, between seasons		
GROUP	F	p
CLT	1.31	0.291
FWT	4.30	0.029

Individual animals of both groups showed a wide random variation in S:P ratio. The lowest S:P ratio in both groups was observed in winter (August) and the biggest difference between groups in summer. A significant difference between FWT and CLT sheep was observed during summer (November) with FWT showing a higher S:P ratio than CLT sheep. FWT sheep showed a significant seasonal change in S:P ratio, this difference being attributed mainly to the observed low ratio in August and high ratio in November. No seasonal effect was seen in CLT sheep.

Activity of follicle population

Table 4.10 **Percentage of anagen follicles of individual animals**

(*: denotes missing data, page: 4.6)

GROUP	IDENTIFICATION NUMBER	JUNE	AUGUST	NOVEMBER
FWT	26	98.6	96.4	100
	28	100	100	100
	37	97.4	100	100
	39	98.4	93.2	100
	42	82.6	74.4	100
	72	93.6	100	100
	73	100	*	100
	120	97.2	*	100
	199	99.2	*	100
CLT	51	100	93.4	100
	147	99.2	97.8	100
	159	100	98.4	100
	182	97.4	*	*
	185	100	100	100
	186	100	97.6	100
	190	100	*	100
	203	100	*	100
	204	81.8	83.8	99.2
	247	*	*	100

Table 4.11 **Group means of the percentage of anagen follicles between seasons**

MONTH	GROUP	N	MEAN	SEM
JUNE	CLT	9	97.6	2.00
	FWT	9	96.33	1.84
AUGUST	CLT	6	95.17	2.44
	FWT	6	94.00	4.08
NOVEMBER	CLT	9	99.91	0.09
	FWT	9	100.0	0.00

Table 4.12 **Significance levels for analysis of variance of mean values of the percentage of anagen follicles**

[A] between groups, within seasons		
MONTH	F	p
JUNE	0.22	0.647
AUGUST	0.06	0.811
NOVEMBER	1.00	0.322

[B] within groups, between seasons		
GROUP	F	p
CLT	1.85	0.186
FWT	1.96	0.165

A small percentage of inactive follicles was seen in most animals of either group during winter (June, August). Of the FWT sheep, No 28 had no catagen follicles, whereas No 42 showed the highest percentage of inactive follicles of all animals examined during the winter months. All follicles of FWT sheep in summer were fully active.

More CLT sheep had 100% follicular activity during June than FWT sheep. Sheep No 185 showed no changes in follicle activity during the entire observation period, whereas No 204 did not reach full activity even during summer. In animals of both groups, catagen was generally observed in secondary follicles and only occasionally in primary follicles. CLT sheep had a higher percentage of active follicles than FWT sheep in winter (June, August) but a slightly lower percentage in early summer (November) (Table 4.11).

No significant differences were seen in either the activity levels between groups or within groups during the observation period (Table 4.12, A and B).

Follicle bulb measurements, compared between groups

Sheep and follicle numbers are shown in Table 4.13. The results on follicle bulb measurements will be given first for a comparison between groups at each of the three stages of the year.

Table 4.13 **Number of sheep in each group (FWT and CLT) for each month and numbers of follicle bulbs measured (N) in each month**

	JUNE	AUGUST	NOVEMBER
FWT	n = 8	n = 9	n = 9
CLT	n = 9	n = 8	n = 9
N	235	293	320

Tables 4.14 to 4.20 give the mean values on the number of labelled bulb cells and several follicle bulb measurements of FWT and CLT sheep, with the analysis of variance on significance of the difference between the two groups for June, August and November respectively. Chapter 3 (page: 3.5) gives a detailed description and validations of the measurements undertaken on follicle bulb sections.

Table 4.14 **Mean number of labelled cells in the follicle bulb of FWT and CLT sheep 2 hours after i.c. injection of BrdU, and level of significance for analysis of variance for group differences at three stages of the year**

MONTH	FWT		CLT		F	p
	MEAN	SEM	MEAN	SEM		
JUNE	42.41	1.0227	36.91	0.8120	13.22	0.0003
AUGUST	45.32	0.7714	36.24	0.9032	55.69	0.0001
NOVEMBER	47.19	0.9524	41.89	0.8165	17.61	0.0001

Table 4.15 **Mean values for bulb diameter (μm) for FWT and CLT sheep and level of significance for analysis of variance for group differences at three stages of the year**

MONTH	FWT		CLT		F	p
	MEAN	SEM	MEAN	SEM		
JUNE	139.11	1.8002	128.95	1.6357	14.58	0.0002
AUGUST	129.25	1.5483	121.56	1.6882	11.38	0.0008
NOVEMBER	156.57	1.6760	147.14	1.6788	18.57	0.0001

Table 4.16 **Mean values for bulb area (μm^2) and level of significance for analysis of variance for group differences at three stages of the year**

[A] area under the line through top of papilla

MONTH	FWT		CLT		F	p
	MEAN	SEM	MEAN	SEM		
JUNE	10934.33	336.3	8942.01	258.6	21.05	0.0001
AUGUST	9658.25	231.6	8056.33	275.9	20.05	0.0001
NOVEMBER	14472.57	390.4	13525.26	402.6	5.72	0.0174

[B] area under the line through labelled cell highest up the bulb

MONTH	FWT		CLT		F	p
	MEAN	SEM	MEAN	SEM		
JUNE	9972.90	241.34	8198.00	195.36	28.15	0.0001
AUGUST	8965.98	181.92	7870.97	182.62	17.07	0.0001
NOVEMBER	12636.44	235.52	12228.71	258.76	2.25/NS	0.1341

Table 4.17 **Mean values for papilla area (μm^2) and significance levels for analysis of variance for group differences at 3 stages of the year**

MONTH	FWT		CLT		F	p
	MEAN	SEM	MEAN	SEM		
JUNE	2015.81	96.33	1691.89	78.82	5.07	0.0253
AUGUST	1873.66	70.82	1602.58	78.76	6.65	0.0104
NOVEMBER	2151.32	98.38	1813.62	84.79	9.30	0.0025

Table 4.18 **Mean values for papilla length (μm) and significance level for analysis of variance for group differences at three stages of the year**

MONTH	FWT		CLT		F	p
	MEAN	SEM	MEAN	SEM		
JUNE	96.41	1.91	87.25	1.59	12.69	0.0004
AUGUST	92.00	1.38	81.56	1.75	22.01	0.0001
NOVEMBER	115.55	2.08	114.03	2.27	1.27/NS	0.2620

Table 4.19 **Mean values for the germinative tissue area (bulb area [A] - dermal papilla area) (μm^2)and significance levels for analysis of variance for group differences at three stages of the year**

MONTH	FWT		CLT		F	p
	MEAN	SEM	MEAN	SEM		
JUNE	8835	263	7247	395	10.59	0.005
AUGUST	7783	263	6438	363	9.50	0.008
NOVEMBER	12331	365	11738	467	1.00	0.33/NS

Table 4.20 **Mean values for diameter of fibre cortex (μm) at the base of the fibre and significance level for analysis of variance for group differences at three stages of the year**

MONTH	FWT		CLT		F	p
	MEAN	SEM	MEAN	SEM		
JUNE	116.00	1.73	110.04	1.76	4.78	0.0298
AUGUST	110.79	1.45	107.06	1.51	3.46	0.0640
NOVEMBER	128.05	2.04	121.03	2.13	5.25	0.0226

Table 4.21 **Mean values for diameter of fibre cortex plus IRS (μm) at the base of the fibre and significance level for analysis of variance for group differences at three stages of the year**

MONTH	FWT		CLT		F	p
	MEAN	SEM	MEAN	SEM		
JUNE	133.43	2.27	126.92	2.12	4.94	0.0272
AUGUST	126.53	1.66	123.37	1.91	1.13/NS	0.2878
NOVEMBER	147.59	1.72	149.29	6.44	0.07/NS	0.7928

FWT sheep had significantly more proliferating cells than CLT sheep in each month especially in August (Table 4.14). At all three stages, FWT retained a wider bulb diameter than CLT, this difference being highly significant (Table 4.15).

Bulb area was measured in two different ways, based on a different definition of the upper area boundary. In Table 4.16 [A], a line through the top of the papilla defined the uppermost boundary of the bulb area. In Table 4.16 [B], the proliferating cell found highest up the bulb toward the fibre was taken as a reference point for the boundary line. Both area measurements include the papilla. FWT sheep had a significantly greater bulb area than CLT sheep at each stage (Table 4.16 [A]). The biggest difference occurred during June. Table 4.16 [B], shows similar results; the only difference was that the advantage of FWT over CLT sheep was no longer significant during summer. Measurements [A] and [B] were highly correlated (r=0.72). [A] is influenced by the papilla length. Details on the description, correlation and validity of different measurements have been given in Chapter 3.

Table 4.17 analyses the papilla areas for FWT and CLT sheep. FWT had larger papilla areas than CLT sheep, this difference being always significant. FWT exhibit the biggest advantage during November, the smallest in August. FWT sheep had a longer papilla than CLT, this difference being most pronounced in August but it was not statistically significant in November (Table 4.18). Mean values for calculated germinative tissue area showed that FWT had significantly larger areas in June and August, but not in November (Table 4.19).

The advantage of FWT sheep in the diameter of the cortex was statistically significant only in June and November. In August the difference was marginal (Table 4.20). With the IRS included, FWT showed a significantly wider diameter than CLT in June. Such a difference was not apparent in August and November (Table 4.21).

Follicle bulb measurements, compared between stages of the year

Analysis of variance F-values and significance levels for the seasonal effect on measured variables are given in Tables 4.22 to 4.28. All variables, except for the number of labelled cells (NO) and the the papilla area (PA) showed a significant seasonal effect with low values in winter and high values in summer. The number of observations for the statistical analysis was 42.

Table 4.22 **Significance levels for the seasonal differences in the number of labelled cells in the follicle bulb for FWT and CLT sheep.**

	CLT	June	August	November
FWT				
June			0.758	0.1593
August		0.2849		0.0091
November		0.1560	0.7141	

Table 4.23 **Significance levels for seasonal differences in bulb diameter measurements of FWT and CLT sheep.**

	CLT	June	August	November
FWT				
June			0.0237	0.0001
August		0.0010		0.0001
November		0.0001	0.0001	

Table 4.24 **Significance levels for seasonal differences in bulb area measurements for FWT and CLT sheep. [A] area is that under a line through the top of the papilla.**

	CLT	June	August	November
FWT				
June			0.2107	0.0001
August		0.0418		0.0001
November		0.0001	0.0001	

[B] area is that under a line through the uppermost labelled cell.

	CLT	June	August	November
FWT				
June			0.760	0.0001
August		0.0057		0.0001
November		0.0001	0.0001	

Table 4.25 **Significance levels for seasonal differences in dermal papilla area for FWT and CLT sheep.**

	CLT	June	August	November
FWT				
June			0.7383	0.7655
August		0.3817		0.5284
November		0.7517	0.2376	

Table 4.26 **Significance levels for seasonal differences in dermal papilla length for FWT and CLT sheep.**

	CLT	June	August	November
FWT				
June			0.216	0.0001
August		0.2539		0.0001
November		0.0001	0.0001	

Table 4.27 **Significance levels for seasonal differences in fibre cortical width for FWT and CLT sheep.**

	CLT	June	August	November
FWT				
June			0.2440	0.0326
August		0.0515		0.0020
November		0.0037	0.0001	

Table 4.28 **Significance levels for seasonal differences in width of fibre cortex plus IRS for FWT and CLT sheep.**

	CLT	June	August	November
FWT				
June			0.507	0.0002
August		0.109		0.0001
November		0.0118	0.0002	

A significant seasonal effect in the differences of the number of labelled cells could not be seen in either line. August nad November differences were significant in CLT sheep (Table 4.22). Bulb diameter changes reached significance at each stage in both groups (Table 4.23). Both bulb area measurements were always significant in their differences between months in FWT sheep. CLT sheep did not reach significance between June and August (Table 4.24, A and B).

Although mean values for dermal papilla area (Table 4.17) show a seasonal change in area with a decrease in size during winter, these differences were not significant in either FWT or CLT sheep (Table 4.25). Papilla length differences were always significant except for FWT and CLT sheep between June and August (Table 4.26).

The cortical diameter and cortical plus IRS width both showed significant differences except in FWT and CLT sheep between June and August (Tables 4.27 and 4.28).

Distribution of bulb matrix cells to cortex and IRS

Table 4.29 **Mean values of cross-sectional area of the fibre cortex at the base of the fibre (μm^2) and significance levels for analysis of variance for the group differences at three stages of the year**

MONTH	FWT		CLT		p
	MEAN	SEM	MEAN	SEM	
JUNE	10655	469	9785	401	0.176
AUGUST	9709	334	9142	528	0.36
NOVEMBER	12866	541	11570	411	0.074

Table 4.30 **Mean values of cross-sectional area of IRS at the base of the fibre (μm^2) and significance levels for analysis of variance for group differences at three stages of the year**

	FWT		CLT		p
	MEAN	SEM	MEAN	SEM	
JUNE	3279	482	3122	432	0.811
AUGUST	2938	231	3089	368	0.722
NOVEMBER	4337	445	6108	157	0.0295

Table 4.31 **IRS area expressed as percent of IRS plus cortex area for FWT and CLT sheep at three stages of the year**

MONTH	FWT	CLT
JUNE	23.9%	24.8%
AUGUST	23.2%	24.6%
NOVEMBER	24.8%	34.3%

The area of cortex and cortex plus IRS was calculated. Subtraction revealed the area of IRS alone. FWT showed larger cortex areas, but only in June a bigger IRS area. In August and November, CLT sheep had larger IRS areas. Expressing IRS area as percent of IRS plus cortex area, a constant proportion of approximately 24% was occupied by the IRS in all three months. This percentage increased to 34% in CLT sheep during November. Only the area of the cortex plus IRS in November approached a level of significant group difference.

Density ratio - derived variables

When the numbers of replicating cells were expressed relative to the area of the replicating zone (proliferation density), the difference between FWT and CLT sheep was no longer significantly different (Tables 4.32 and 4.33).

Table 4.32 **Least square means of ratio of the number of replicating cells per area of replicating zone for FWT and CLT sheep with the level of significance for the difference in group effect at three stages of the year**

		JUNE	AUGUST	NOVEMBER
significance level		**0.53**	**0.92**	**0.88**
CLT	LSMEAN	0.0055	0.0064	0.0040
	SEM	0.0002	0.0003	0.0003
FWT	LSMEAN	0.0052	0.0063	0.0042
	SEM	0.0002	0.00015	0.0006

Table 4.33 **Analysis of variance table of General Linear Model Procedure for month and group effect on the proliferating density ratio.** Number of observations: 42

Source	DF	F	p
Month	2	24.62	0.0001
Group	1	0.26	0.6126
Group x Month	2	0.99	0.9177
Animal (Group)	12	1.12	0.3898

However, the proliferation density ratio was highly significantly different between months, with the highest ratio in August and the lowest in November for both lines.

General model for seasonal pattern in the morphological behaviour of follicle structures

When the measurements at each time of the year were combined into one table, replacing the absolute values with scores as fractions of 1.0 (allocated to the highest FWT measurements in November) a distinct pattern became apparent.

All, except the number of labelled cells in both FWT and CLT sheep showed an intermediate value in June, a lowest value in August and a highest value in November. This pattern was consistent for follicle dimensions as well as for fibre dimensions and follicle population variables (S:P ration, percentage of anagen follicles). The pattern for fibre length growth rates, since they were measured only once during winter, was assumed to follow the same pattern as other measurements during winter. However, the number of proliferating cells in FWT sheep had its minimum in June and started increasing in August to reach its highest value in November. In CLT sheep, June and August showed an equally low number of proliferating cells (Figure 4.1). For all measured variables, except DI and the percentage of active follicles (%), the highest measurement recorded for CLT sheep in November was lower than the corresponding measurement of FWT sheep.

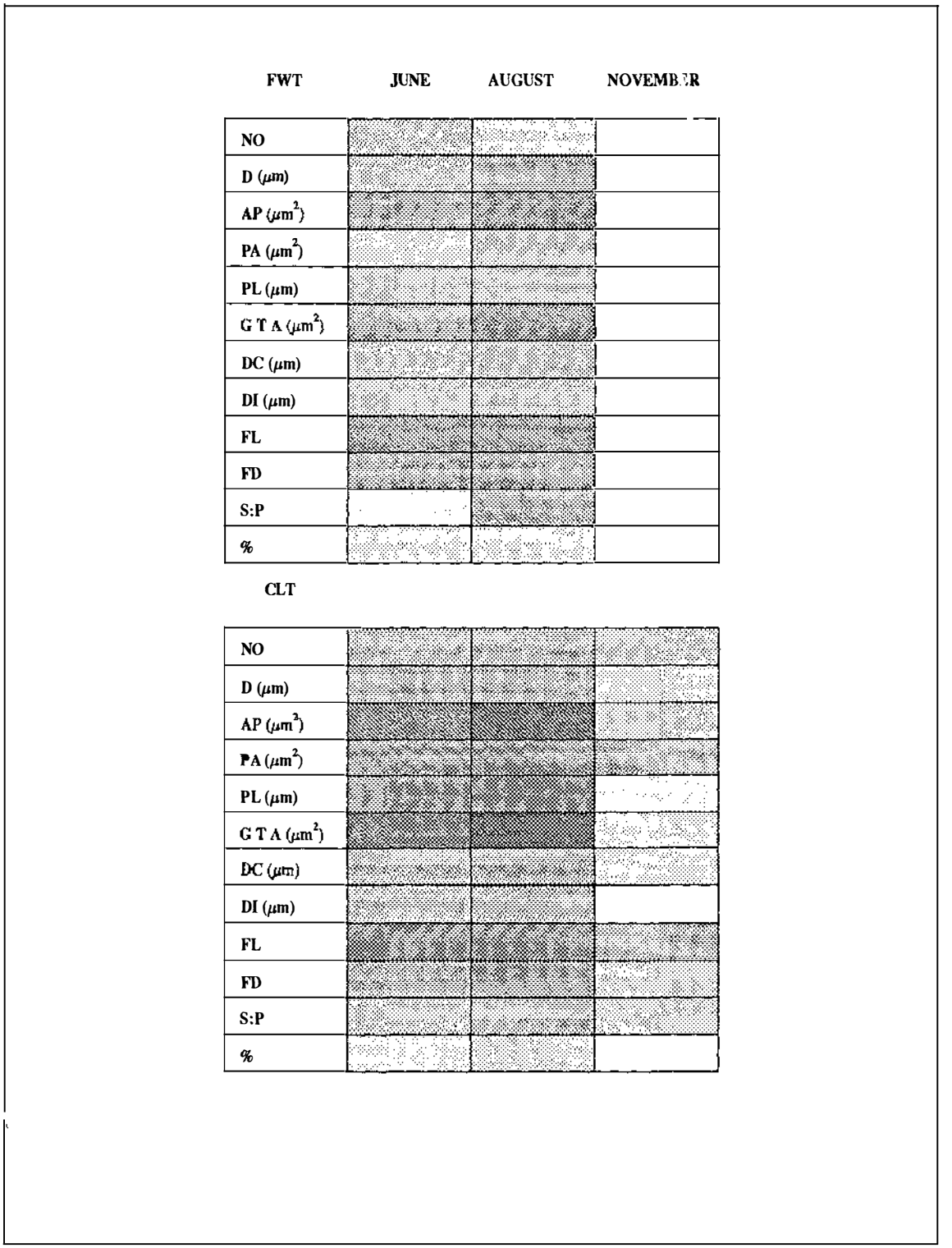

Figure 4.1 **Score chart of follicle and fibre measurements obtained in FWT and CLT sheep at three stages of the year. White boxes indicate the maximum score (1.0), all others are fractions of 1.0 represented in different shades of grey, according to their magnitude related to FWT sheep November sample.** D=bulb diameter, GTA=germinative tissue area, %=percentage of active follicles.

Replication rate of follicle bulb cells

In one month (November), the number of labelled S-phase cells was counted in samples taken 2 hours as well as 6 hours after i.c. injection of BrdU, to assess the rate of increase of labelled nuclei after administration of BrdU in both treatment groups, following a suggestion by Clarke (1971).

Table 4.34 **Number of BrdU labelled nuclei for FWT and CLT sheep in November, at 2 and 6 hours after local i.c. injection of BrdU**

		FWT	CLT
Number of labelled cells per bulb section at 2 hours	MEAN	46.99	42.54
	SEM	2.60	1.82
Number of labelled cells per bulb section at 6 hours	MEAN	58.74	52.51
	SEM	3.73	3.81
rate of accumulation of labelled nuclei per bulb section per hour		**2.94**	**2.49**

Table 4.35 **Significance level for analysis of variance for differences in number of labelled bulb cell nuclei between 2 and 6 hour samples in FWT and CLT sheep**

GROUP	N	F	p
FWT	18	6.69	0.02
CLT	16	5.58	0.03

Table 4.36 **Significance level for analysis of variance for differences between FWT and CLT sheep in the number of labelled bulb cell nuclei in 2 and 6 hour samples and the rate of accumulation per hour**

	N	F	p
2 hours	17	1.88	0.191
6 hours	17	1.36	0.262

In both groups, nucleus counts were significantly higher in the 6 hour samples than in the 2 hour samples, with differences of 11.76 cells for FWT and 9.98 cells for CLT sheep respectively (Tables 4.34 and 4.35). Expressed as rate of hourly increase of labelled cells, FWT had an advantage of 18%.

Percentage advantage of FWT over CLT sheep

Table 4.37 **Differences between FWT and CLT sheep in follicle measurements, including the germinative tissue area (GTA), expressed as percentage advantage of FWT over CLT sheep (FWT-CLT/CLT x 100)**

VARIABLES	JUNE	AUGUST	NOVEMBER
NO	14.9%	25.1%	12.7%
DS	7.87%	6.33%	6.41%
AP	22.3%	19.9%	7.00%
PA	19.2%	16.9%	18.6%
PL	10.5%	12.8%	1.30%
DC	5.42%	3.48%	5.80%
DI	5.28%	2.56%	-1.14%
GTA	21.9%	21.0%	5.05%

Absolute values obtained for follicle measurements on FWT and CLT sheep were expressed as percentage advantage of FWT over CLT sheep (Table 4.37). The percentage advantage showed strong seasonal variations especially for NO, AP and the area of germinative tissue. In August NO accounts for all of the 25% flock difference reported by McClelland et al. (1987b), AP accounted for almost 20% and the germinative tissue area for 21%. A striking change could be seen in November, the area AP only accounting for 7%, the germinative tissue area for 5% and NO for 12.7%. DS, PA and DC appeared to be the most consistent. The increase of BrdU labelled cells between 2 and 6 hours in November accounted for 18% of between line difference.

Correlation of fibre and follicle dimensions

The different follicle variables taken in June, August and November were individually regressed on fibre length and fibre diameter for both treatment groups. A student t-test was added to test for the significance of differences of the regression coefficients between months for each variable. Where monthly changes between relationships were non-significant, regressions of combined data for all three months were made. Follicle variables measured in June were matched to June fibre diameter measurements and winter (June to August) fibre length growth measurements. August skin samples were matched with September fibre diameter measurements and winter (June to August) fibre length growth measurements. November skin samples were

matched to December fibre diameter and summer (November to December) fibre length growth measurements.

Fibre Length

Table 4.38 **Analysis of variance values for regression of follicle parameters on fibre length in CLT sheep, R-sq and student t-test for significance of differences between months within individual parameters**

MONTH		JUNE	AUGUST	NOVEMBER	COMBINED
N		6	5	7	18
ANOVA	R-sq	64.5%	2.6%	2.5%	6.5%
NO	F	7.28	0.08	0.13	1.11
	p	0.054	0.794	0.737	0.308
		NS	NS	NS	NS
student t-test		NS			
			NS		
ANOVA	R-sq	29.7%	58.1%	13.1%	30.1%
PA	F	1.69	4.17	0.76	6.90
	p	0.264	0.134	0.424	0.018
		NS	NS	NS	**
student t-test		NS			
			NS		
ANOVA	R-sq	34.7%	67.1%	44.4%	20.6%
PL	F	2.13	6.11	3.99	4.16
	p	0.218	0.09	0.102	0.058
		NS	NS	NS	NS/marg.
student t-test		NS			
			NS		

MONTH		JUNE	AUGUST	NOVEMBER	COMBINED
N		6	5	7	18
ANOVA	R-sq	69.2%	57.9%	15.0%	27.1%
DS	F	9.0	4.13	0.88	5.96
	p	0.04	0.135	0.391	0.027
		**	NS	NS	*
student t-test		NS			
			NS		
ANOVA	R-sq	52.6%	57.8%	43.6%	24.5%
AP	F	4.43	4.11	3.86	5.2
	p	0.103	0.136	0.106	0.037
		NS	NS	NS	*
student t-test		NS			
			NS		
ANOVA	R-sq	31.5%	47.3%	36.8%	31.3%
DC	F	1.84	2.70	2.91	7.28
	p	0.247	0.199	0.149	0.016
		NS	NS	NS	*
student t-test		NS			
			NS		
ANOVA	R-sq	49.5%	38.1%	1.9%	12.8%
DI	F	3.92	1.85	0.10	2.35
	p	0.119	0.267	0.768	0.145
		NS	NS	NS	NS
student t-test		NS			
			NS		

Table 4.39 **Analysis of variance values for regression of follicle parameters on fibre length in FWT sheep, R-sq and student t-test for significance of differences between months within individual parameters**

MONTH		JUNE	AUGUST	NOVEMBER	COMBINED
N		7	7	5	19
ANOVA	R-sq	4.8%	43.2%	0.0%	6.8%
NO	F	0.25	3.80	0.00	1.32
	p	0.638	0.109	0.999	0.266
		NS	NS	NS	NS
student t-test		NS			
			NS		
ANOVA	R-sq	29.3%	30.9%	1.1%	1.1%
PA	F	2.07	2.23	0.03	0.19
	p	0.21	0.195	0.864	0.665
		NS	NS	NS	NS
student t-test		NS			
			NS		
ANOVA	R-sq	19.6%	22.0%	3.4%	15.0%
PL	F	1.22	1.70	0.11	3.16
	p	0.32	0.241	0.765	0.092
		NS	NS	NS	NS/marg.
student t-test		NS			
			NS		
ANOVA	R-sq	0.5%	10.2%	8.8%	14.8%
DS	F	0.02	0.68	0.29	3.12
	p	0.883	0.442	0.628	0.095
		NS	NS	NS	NS/marg.
student t-test		NS			
			NS		

MONTH		JUNE	AUGUST	NOVEMBER	COMBINED
N		7	7	5	19
ANOVA	R-sq	0.1%	21.7%	4.5%	15.3%
AP	F	0.01	1.66	0.14	3.26
	p	0.946	0.245	0.732	0.088
		NS	NS	NS	NS/marg.
student t-test		NS			
			NS		
ANOVA	R-sq	1.5%	2.4%	14.3%	5.9%
DC	F	0.06	0.15	0.5	1.12
	p	0.817	0.715	0.531	0.303
		NS	NS	NS	NS
student t-test		NS			
			NS		
ANOVA	R-sq	10.1%	9.6%	3.8%	9.1%
DI	F	0.45	0.64	0.12	1.80
	p	0.539	0.454	0.754	0.197
		NS	NS	NS	NS
student t-test		NS			
			NS		

In CLT sheep only follicle diameter in June appeared to be significantly related to fibre length growth rates (Table 4.38). All other parameters had low relationships never reaching significance. In FWT sheep there were no apparent relationships (Table 4.39). A trend towards closer relationships in winter was apparent in both FWT and CLT sheep.

Fibre Diameter

Table 4.40 **Analysis of variance values for regression of follicle parameters on fibre diameter in CLT sheep, R-sq and student t-test for significance of differences between months within individual parameters**

MONTH		JUNE	AUGUST	NOVEMBER	COMBINED
N		7	4	7	18
ANOVA	R-sq	37.0%	74.8%	15.9%	-
NO	F	2.93	5.93	0.94	-
	p	0.147	0.135	0.376	-
		NS	NS	NS	-
student t-test		NS			
			2.037/*		
ANOVA	R-sq	28.0%	31.8%	46.6%	32.1%
PA	F	1.94	0.93	4.36	7.55
	p	0.222	0.436	0.091	0.014
		NS	NS	NS/marg.	*
student t-test		NS			
			NS		
ANOVA	R-sq	19.9%	97.4%	37.8%	-
PL	F	1.24	73.63	3.04	-
	p	0.316	0.013	0.141	-
		NS	*	NS	-
student t-test		3.7294/**			
			3.9587/**		
ANOVA	R-sq	68.6%	19.7%	35.5%	59.5%
DS	F	10.93	0.49	2.75	23.51
	p	0.021	0.556	0.158	0.000
		*	NS	NS	**
student t-test		NS			
			NS		

ANOVA	R-sq	42.3%	72.0%	52.2%	56.9%
AP	F	3.67	5.14	5.46	21.1
	p	0.113	0.151	0.067	0.000
		NS	NS	NS/marg.	***
student t-test		NS			
			NS		
ANOVA	R-sq	66.4%	6.5%	48.5%	50.3%
DC	F	9.87	0.14	4.71	16.18
	p	0.026	0.745	0.082	0.001
		*	NS	NS/marg.	***
student t-test		NS			
			NS		
ANOVA	R-sq	85.1%	4.3%	2.2%	29.0%
DI	F	28.63	0.09	0.11	6.52
	p	0.003	0.792	0.749	0.021
		**	NS	NS	*
student t-test		NS			
			NS		

Table 4.41 **Analysis of variance values for regression of different follicle parameters on fibre diameter of FWT, R-sq values and student t-test for significant differences of regression coefficients between months within one parameter on fibre diameter**

MONTH		JUNE	AUGUST	NOVEMBER	COMBINED
N		7	6	6	19
ANOVA	R-sq	2.0%	13.0%	80.8%	0.9%
NO	F	0.10	0.6	16.87	0.16
	p	0.762	0.483	0.015	0.692
		NS	NS	**	NS
student t-test		NS			
			NS		

MONTH		JUNE	AUGUST	NOVEMBER	COMBINED
N		7	6	6	19
ANOVA	R-sq	0.0%	89.7%	0.1%	-
PA	F	0.00	34.84	0.00	-
	p	0.993	0.004	0.957	-
		NS	***	NS	
student t-test		39.76/***			
			53.057/***		
ANOVA	R-sq	5.3%	50.9%	0.3%	51.1%
PL	F	0.28	4.08	0.01	17.79
	p	0.618	0.111	0.923	0.001
		NS	NS	NS	***
student t-test		NS			
			NS		
ANOVA	R-sq	0.8%	50.5%	12.3%	53.7%
DS	F	0.04	4.08	0.56	19.71
	p	0.852	0.114	0.496	0.000
		NS	NS	NS	***
student t-test		NS			
			NS		
ANOVA	R-sq	17.5%	58.6%	2.3%	50.0%
AP	F	1.06	5.66	0.10	16.98
	p	0.35	0.076	0.776	0.001
		NS	NS/marg.	NS	***
student t-test		2.61/*			
			2.243/*		

MONTH		JUNE	AUGUST	NOVEMBER	COMBINED
N		7	6	6	19
ANOVA	R-sq	2.8%	0.0%	2.7%	32.0%
DC	F	0.14	0.00	0.11	8.28
	p	0.72	0.998	0.757	0.01
		NS	NS	NS	*
student t-test		NS			
			NS		
ANOVA	R-sq	0.1%	46.4%	30.1%	47.0%
DI	F	0.00	3.47	1.72	15.05
	p	0.959	0.136	0.259	0.001
		NS	NS	NS	***
student t-test		NS			
			NS		

For CLT sheep, a moderate relationship generally occurred between fibre diameter and all variables, but most did not reach significance level. The strongest relationships were seen for NO, PL and AP in August, DS, DC,and DI in June, with PL, DS, DC and DI reaching significance. Strong significant combined relationships occurred for DS, AP and DC. Significant seasonal changes in the degree of relationship was seen in NO and PL. Three variables (NO, PA, AP) show strongest relationships in August and weakest in November, but generally no overall pattern in the degree of relationship is apparent.

For FWT sheep, variables were generally less strongly related to fibre diameter. Several variables showed almost no relationship. A repeated observation with FWT sheep was the occurrence of inverse relationships during certain periods of the year, chiefly in June, with all measured follicle parameters including the number of labelled cells (November). Except for the latter, these inverse relationships were never significant and never strong (17.5%-0.0%). The number of labelled cells in November though, was 80.8% with a significance level of $p<0.01$. Highest relationships were seen for NO in November, PA, PL, DS, AP and DI in August. DC was only weakly related to fibre diameter. Strong combined relationships were seen for PL and DS. PA and AP showed significant seasonal changes in their relationship for fibre diameter.

Overall, FWT sheep showed a pattern with strong relationships occurring in August and weak relationships in June. This is different to CLT sheep, which did not exhibit a strong pattern. The only similarity between the regression of variables in FWT and CLT sheep was a similar combined strong significant relationship to DS.

DISCUSSION

Fibre length and diameter measurements

Both fibre length growth rates and fibre diameter in New Zealand Romney sheep are subject to seasonal variations with minimum values occurring in winter and maximum values in summer (Ross, 1965; Story and Ross, 1960). In contrast, the Merino sheep appears to be influenced mainly by nutrition rather than seasonal variations (Doney, 1966). The present results showed a seasonal pattern in length growth rates, with a winter decline to CLT levels for FWT sheep, so that differences between lines only occurred in summer. Fibre diameter exhibited a winter decline in both lines, but again it was only in summer that a significant line difference was apparent. According to data of McClelland et al. (1987b) the percentage difference of FWT to CLT sheep in clean wool production per day was greatest during winter, but the greatest absolute difference occurred in summer. Liu (1991) stated that fibre length accounted for most of the between line differences in the same two flocks. From results in the present study, it appears that FWT attain their superiority, at least for the fibre production parameters, by means of a higher growth in summer. With CLT sheep showing a less marked seasonal rhythmic pattern, the greater productivity of FWT sheep might well, in addition to a greater production level, be achieved through a greater amplitude in their seasonal cycle, due to a greater susceptibility to seasonal influences.

The difference between the present results, and previous results reported by McClelland et al. (1987b) on the same selection lines, could be due to the problems of sampling the population in different years. The individual variation in sheep possibly overrides the flock effect. Furthermore, the possibility of different seasonal effects in different years might be due to a lack of repeatability between seasons. Seasonal effects include the response to photoperiod as well as to nutritional supply and to other environmental influences, therefore effects due to genotype could be partly obscured by changing environmental or nutritional influences.

On the other hand, measuring these traits at three times of the year expecting to target extreme periods of either high or low production is still prone to a great scatter of values due to within and between animal variation in performance (Woods and Orwin, 1988). Differences

in the timing and amplitude of the seasonal cycles of each measured parameter can partly eliminate the expression of the between flock differences.

S:P ratio

Follicle density has been claimed to be part of the selection response of increased fleeceweight in Merinos (Williams and Winston, 1987). Follicle density is positively correlated to S:P ratio (Lyne and Hollis, 1968; Jackson et al., 1975). For these reasons, the present study concentrated on S:P ratio only, in order to characterize follicle population densities.

S:P ratio differed between lines only in summer, and only FWT sheep showed a seasonal pattern, a result corresponding to observations on fibre traits. Increased follicle density might well be a specific feature of the Merino, where selection criteria demand a ceiling on fibre diameter (Turner et al., 1968; Turner and Jackson, 1978), in which case increased growth must be brought about chiefly through fibre length growth and follicle density changes. These mechanisms have already been discussed in Chapter 1 (page: 1.1). According to the concept of competition between adjacent hair follicles (Fraser and Short, 1960), density and fibre diameter of the fleece are highly negatively correlated, with the diameter being significantly affected by the number of adjacent follicles, because of competition for nutrients. It appears, however, that the follicle population of FWT sheep is not yet dense enough to exert that form of competition. A simultaneous increase in both density and fibre diameter is therefore possible.

Activity of the follicle population

Observed fluctuations in the percentage of active follicles within the population could not be attributed to either seasonal influence or treatment line effect. This suggests that the cycle of wool follicle activity (anagen-catagen) is not under seasonal influence in these animals but is intrinsic to the individual follicles. Selective breeding for heavier fleece production appears to have no effect on the wool growth cycle. The effects of selective breeding on follicle activity in Merino sheep does not appear to have been studied, but, in Merinos, when showing a response to nutrition, Short et al. (1965) observed a very small decrease in the percentage of inactive follicles. Again, in Merinos where density is one of the main traits responding to wool production changes (Hocking Edwards and Hynd, 1992), the fleece could have reached a density where follicles are very much in competition for available nutrients. Small declines in nutrient supply could reflect in some follicles not being able to retain cell proliferation due to energy shortage. Consequently these follicles could degenerate into the inactive state until nutrient

supply increases again.

Follicle bulb measurements, flock effect

From the results of several different follicle bulb measurements, it is concluded that FWT sheep show advantages over CLT sheep by a greater number of proliferating cells as well as by greater bulb and dermal papilla size.

Follicle bulb measurements, seasonal effect

Both lines show a very significant seasonal effect on follicle measurements with low values occurring in winter and high values in summer. Only the number of proliferating cells and the dermal papilla area was independent of seasonal influences. The results suggested that overall dermal papilla size is brought about by changes in papilla length, where there was a significant seasonal effect. This is in agreement with the one third depression of papilla height during winter reported by Rudall (1956).

Differences in the significance levels of the two bulb area measurements are thought to be due to difference in definition of their distal boundaries. The bulb area [A] has the dermal papilla as a significant component, hence the significance in the results in [A] could be partly caused by the influence of the dermal papilla. Details of and comments about the design and significance of measured parameters are given in Chapter 3 (page: 3.5).

FWT sheep appeared to have a larger proliferating cell area, at least during winter, an observation in accord with findings of Williams and Winston (1987) in Merino sheep ("Fleece Plus"). The amount of germinative tissue can be influenced by seasonal changes in Romney sheep, accompanied with a corresponding change in the number of labelled cells. This confirms the observations of Short et al. (1965).

Bulb area was shown in Chapter 3 (page: 3.14) to be strongly related to papilla area and papilla length, a finding suggesting the importance of the dermal papilla as an influence on hair growth. Cohen (1965) postulated that, in an established follicle, the dermal papilla (size) determines the number of mitoses in the follicle bulb matrix.

Density ratio

When the advantage of FWT sheep over CLT sheep was expressed as a ratio of replicating cells per area of germinative tissue in the follicle bulb, the between line differences disappeared.

If, as described in Chapter 3 (page: 3.16), follicles of FWT sheep possess more cells in their larger germinative tissue areas, it is possible that they also show a greater number of proliferating cells. This does not necessarily mean that the proliferative/mitotic density is greater, as shown by the results obtained in the present study. Thus, if other control mechanisms influencing the maturing fibre are kept constant, the size change appears to control the net output of fibre.

As outlined in Chapter 3 (page: 3.26), only the ratio presented here (number of proliferating cells per area germinative tissue (ASC)) achieved a non-significant result for between line comparisons. Ratios with other variables (papilla length or bulb diameter) showed varying results. Hence, the conclusion concerning density ratio in the present study has to be considered cautiously.

Cell proliferation rates in the follicle bulb

There are several possibilities as to how the number of proliferating cells, and the rate at which these cells proliferate, could affect overall cell production. An equal number of proliferating cells maintain production by a similar or changed rate (faster or slower) or, a smaller number of proliferating cells can produce at the same, or with a changed rate. In the present study, where a greater number of proliferating cells was observed in FWT sheep, these cells might also vary in proliferation rate. In case of rate acceleration, FWT advantage will be enforced, in case of rate decline FWT advantage will be diminished.

Line differences in the accumulation of labelled cells from 2 to 6 hours after local i.c. injection of BrdU were not significant. The hourly rate of cell accumulation, however, expressed as percentage advantage of FWT over CLT sheep, accounted for 18% of the group difference. This result suggests that an increased rate could be a factor aiding toward increased wool production in FWT sheep at least in summer, when the contribution of NO, AP and germinative area declines in comparison to that in winter (Table 4.37). Since rate processes in the follicle bulb were not investigated in detail in the present study, further research should be conducted clarifying the contribution of possible rate changes in the follicle matrix cells of the selection lines.

The measured production of approximately 2 new cells per follicle bulb section per hour

confirms the findings reported in Chapter 2 (page: 2.14). Preliminary work on three dimensional reconstruction of the follicle bulb measurements supports that this is equivalent to approximately 25-30 new cells per bulb per hour, which is in the same order of magnitude as that reported by Hocking Edwards and Hynd (1992).

Distribution of bulb matrix cells to cortex and IRS

In this study, different genotypes showed variations in width as well as area of cortex and IRS, measured at a level across the top of the dermal papilla. The expression of these differences was further enhanced through seasonal influences. On the assumption that cells pass from the bulb into the IRS at the same rate as into the cortex, and assuming no differences in cell size between cells in the bulb and cells at the level where cortex and IRS areas were measured, these results demonstrate that the lines differ in cell distribution. This supports the suggestion by Hynd (1989a, b) that differences in the distribution of cells to either cortex or IRS exist and are inherent. Furthermore, the results obtained in the present study suggest that apart from genetic control, an additional environmental or seasonal component exists influencing cell redistribution.

Short et al. (1965) reported a proportion of only 45% being occupied by the cortex and the larger proportion of 55% by the IRS in the Merino. The results from the current study, based on measurements undertaken at approximately the same level at the top of the bulb as those of Short et al., showed that the cortical area occupied 70-80%. Under the assumptions mentioned above, the majority of bulb cells in the Romney appear to contribute to the cortex. Short et al. (1965) calculated that only 20% of bulb matrix cells eventually appear in the fibre, while Hocking Edwards and Hynd (1992) calculated 20-60% depending on the genotype. In this study, no calculations at the fibre level were made. However, a three-dimensional reconstruction of double BrdU labelled cell populations (6 and 72 hours) in the bulb and the shaft of the same sheep used in this study, showed about 40% of the number of labelled bulb cells appearing in the shaft (K. Kelly et al., personal communication). This result is in agreement with the calculations by Hocking Edwards and Hynd (1992) for the strongwool breeds.

In summary, these observations suggest that genotype primarily determines the proportion of cells forming the cortex and IRS, with finewool breeds distributing markedly less cells into the cortex. However, measurements of the two components (cortex and IRS) undertaken at two different levels (across the top of the dermal papilla and in the shaft) suggest that their magnitude is very much in proportion to each other, with a tendency to decline to half the proportional values in the fibre to that measured at the level of the top of the dermal papilla.

This observation can be partly supported by the close relationship of FD and DS (Table 4.40 and 4.41). If this close relationship could be firmly established in the future, it might be sufficient to measure cortex and IRS areas at top dermal papilla level to deduct results for the fibre.

Relationship of fibre and follicle dimensions

Errors in the measurements

Possible errors introduced, contributing to a weak correlation of parameters, could have resulted from some of the methods of collecting samples and subsequent measurement of variables. Fibre samples and skin samples were taken from the same midside region of the sheep, but fibres could not have originated from exactly the same follicle population present in the skin sections. Differences exist between individual sheep for the same body region (Lyne and Hollis, 1968) accounting for a great part of between animal variation in animals of the same group as well as between groups.

A further source of error in comparing follicle with fibre development could be due to a lack of coordination of events in the seasonal changes of follicle dimensions, fibre diameter changes and fibre length changes. The two latter components were reported to be subject to seasonal changes with a certain degree of independence (Ross, 1965; Story and Ross, 1960) for Romney sheep, with maximum and minimum growth in length preceding maximum and minimum fibre diameter by about a month.

Henderson (1965) reported a high variability of follicle dimensions both within and between populations of follicles as well as a strong relationship of fibre diameter to follicle dimension only within the follicle population of one sheep. He concluded that follicle dimensions had only a small and inconsistent influence on the variability of fibre size in sheep.

Since in the present study no regular measurements were made to evaluate seasonal effects, changes in parameters with delayed or accelerated seasonal cycles could have been missed. Although in general fibre and follicle parameters were poorly correlated (Tables 4.38 to 4.41), a similar overall pattern in their seasonal behaviour was observed (Figure 4.1), suggesting a certain degree of correspondence in their annual rhythms.

Relationship of fibre to follicle measurements

Fibre diameter is related to bulb diameter, cortical diameter and cortical plus IRS diameter, suggesting that although marked diameter changes take place in the width of a fibre from the bulb level to the surface (Orwin and Woods, 1982) they maintain proportionality. Most of the wool production variations in Romney sheep are the result of fibre diameter changes

(Henderson, 1965), while fibre length growth rates have less influence. This observation can explain the result of the present study where relationships of fibre length to follicle parameters were seldom observed.

Changes in fibre production are thus chiefly brought about by changes in the size of the follicle bulb. Germinative tissue area and dermal papilla influence mainly fibre diameter. In addition, the distribution of bulb cells to either cortex or IRS appears to modulate total fibre output, again mainly through fibre diameter changes.

In summary, FWT sheep appear to achieve greater wool growth because they are more responsive to seasonality. In addition to achieving greater productivity through larger follicle dimensions in winter, acceleration of cell proliferation rates in summer further contributes to their genotypic superiority. The dermal papilla of the follicle, as an organ delivering nutrients by diffusion to the bulb, is a likely primary organ in the mediation of fibre growth. Of all measured relationships of fibre to follicle dimensions, the influence of papilla dimensions on fibre diameter was one of the clearest. The general observation that FWT sheep are more responsive to seasonal influences supports a hypothesis that papilla length is a target structure for genetic and environmental influences, by serving as a mediator for subsequent follicle dimensional modulations.

From the results achieved on both lines, the following scheme appears to be the most likely. The germinative tissue area, being absolutely larger in FWT than in CLT sheep, changes proportionally with the dermal papilla area under seasonal influence in FWT sheep. In contrast, the germinative tissue area in CLT sheep enlarges disproportionally more in summer, to an extent that both lines have similar sizes of germinative tissue areas in summer. Other production traits (fibre length growth rates and fibre diameter) are significantly increased in FWT sheep in summer, a result which does not concur with the results on bulb dimensions mentioned above. Higher production in terms of fibre length and fibre diameter can be achieved in two ways:

a) additional rate changes in cell production during summer

b) redistribution of proliferating bulb cells into more cells migrating into the IRS In the case of (a), FWT showed a 18% advantage in hourly cell production in summer at a time when bulb areas only accounted for 5% and 7% of the between line differences. Additionally, they had larger dermal papillae during summer, suggesting an advantage in availability of nutrients and energy for accelerated cell production.

In the case of (b), CLT sheep showing similar germinative tissue areas would still exhibit lower levels of fibre production if relatively more cells were fed into their IRS.

DISCUSSION

THE USE OF BROMODEOXYURIDINE (BrdU) IN STUDYING CELL PROLIFERATION IN WOOL FOLLICLES

This study established an immunocytochemical detection method of in vivo labelling with bromodeoxyuridine (BrdU) suitable to investigate cell proliferation and migration patterns in the wool follicle (Chapter 1). Two different modes of application were investigated; one through infusion into isolated midside skin patches after surgical modification of the animals (Chapter 1) and the other by localised intracutaneous injection (Chapter 2). Intracutaneous injection appears to be superior to vascular infusion because of the ease of injection, making it suitable for field studies. BrdU showed a slow tissue clearance after local i.c. injection. This administration method is therefore similar to a continuous exposure, rather than a pulse exposure. This behaviour has to be taken into account in the design of further experiments. If cells are to be labelled continuously, BrdU only needs to be injected every 24 hours. If it is desired to label different populations, sequential injections need to be more than 24 hours apart.

Despite advantages of BrdU over ^{3}H-thymidine, BrdU (Chapter 2) might interfere with normal cytokinetic and cytodynamic events. The development of a localised administration decreases the injection loads markedly and thus decreases the risk of toxic side effects on the whole body level. This study did not, however, investigate the possibility of long term effects (longer than 24 hours) of local BrdU pools on cellular events in the follicle. Further studies should be conducted to investigate toxic effects after long term exposure to BrdU after single as well as after repeated localised injections into the skin.

Assuming no untoward effects on cell production and differentiation, BrdU would be well suited for further studies on cell migration patterns by repeated injections and double labelling techniques, in combination with other halogenated thymidine analogues such as fluoro- and iododesoxyuridine. Radioactive labelled amino acids (^{35}S-methionine or cysteine) could be applied in parallel to estimate fibre growth concurrently with cell production in the follicle bulb. BrdU, as a label of proliferating cell nuclei, is unsuited for migration studies beyond the point of fibre and IRS keratinization, where cell nuclei disintegrate. Here, radioactive labelled amino acids are more appropriate, since they are retained in the fibre. Direct comparisons of the effects

of locally administered hormones or growth factors (e.g. epidermal growth factor) on cell proliferation could be studied in the future. BrdU could be used to elucidate cellular events in the ORS.

Despite the possibility of applying BrdU as a marker for cell proliferation, such markers are unsuitable for observing the migration of individual cells from the bulb into the fibre or IRS. They do not determine the origin of a labelled cell seen distally. Ideally, individual cell movement in a fully operationable follicle, preferably in vivo, should be monitored. In vitro follicle studies in cultured skin slices (Ward and Harris, 1976) might establish such a technique.

GERMINATIVE TISSUE AREA AND CELL PROLIFERATION IN THE FOLLICLE BULB

Results obtained on the validation of different boundary lines for the germinative tissue area (Chapter 3) revealed that a line across the top of the dermal papilla best represents the germinative tissue area in terms of correlation to other follicle dimensions and cell proliferation. The area defined by Short et al. (1965) did not give satisfactory results.

Proliferating cells could be seen throughout the defined area. The majority of cells are found in the lower two thirds of the bulb, in samples taken 30 minutes as well as in samples taken 2 and 6 hours after injection, well before the onset of migration of these cells. Thus, the position of labelled cells closely coincides with their actual position at the time of proliferation.

If there is a single layer of stem cells around the dermal papilla (Potten, 1978), and if cells of the same generation move distally close together, cells from the lower bulb would have to move with a greater velocity than cells in level with the top of the papilla. Alternatively, the lower bulb cells may be proliferating more by an asymmetrical amplifying scheme, suggested by Potten (1978). Indeed, more proliferating cells are seen in the lower parts of the bulb.

No interpretation of cells moving further up the bulb can be made, since in this study only the lower bulb was investigated. Hence, it would be a challenging aspect for further research to clarify whether the velocity of relative cell movement changes toward the fibre and how this influences the interpretation of results achieved on cell population kinetics in the lower bulb. As already outlined in the general introduction (page: I.21) evidence by Orwin and Woods (1982) on the existence of intercellular bridges between cells in the lower but not the upper bulb supports this aspect.

MECHANISMS CONTROLLING FIBRE GROWTH, INVESTIGATED AS A RESPONSE TO SELECTION FOR INCREASED WOOL PRODUCTION

Variations in the rate of wool growth between animals of either similar or different genotypes reflect the interaction of environmental and genetic factors. These variations occur in sheep as response to photoperiod, weather, nutritional supply and differences in physiological states such as pregnancy or lactation. Under normal nutritional regimen and disregarding physiological extremes, fibre output is primarily determined by genetic factors interacting with the nutrient supply to the follicle. Genetic determinants of wool growth can be expected to be:

- the total number of follicles
- the number (and size) of cells in the proliferative zone of the follicle bulb
- the proliferation rate of the follicle bulb cell population
- the proportion of cells migrating to form the fibre cortex or IRS
- the final size, arrangement and type of cortical cells.

Some of these have been researched in this thesis.

Black (1987) discussed the possibility that the maximum potential for each of these characteristics is genetically controlled, but that the follicle environment influences the expression of actual values. Since conclusive evidence of the genetic control of the maximum potential is not yet firmly established, new information about follicle and fibre characteristics of sheep genetically selected for different levels of wool production adds to the basic understanding of cellular mechanisms controlling wool growth. This, in turn, could enhance the efficiency and quality of wool production.

This study has attempted to establish the effects of genetic selection for high fleece weight in the NZ Romney sheep on the morphology of the individual follicle and the kinetics and dynamics of the germinative cell population in the follicle bulb. The results are summarized and discussed below.

Fibre characteristics

The selection response primarily influenced fibre diameter and to a lesser degree fibre length growth rates. Combined as fibre volume output, selection has produced a positive response.

Skin characteristics

Selection for higher fleeceweight had only a small effect on the ratio of secondary to primary follicles, and consequently on the density of the follicle population. This response was dependent on photoperiod. It had no effect on the proportion of anagen follicles in the population, although in both selection flocks inactive follicles were observed. The latter result is of interest in so far as it showed that, even in sheep breeds not believed to exhibit a distinct annual hair cycle, some follicles are not actively growing. This behaviour may be intrinsic to the individual follicle, rather than being under the influence of photoperiod or selection. It can be regarded as remnant of the original shedding mechanism seen in unimproved breeds. The factors triggering the degeneration of follicles in improved breeds are not known.

The finding that the S:P ratio is higher in summer, is unlikely to be evidence that in each summer secondary follicles grow anew. For the estimation of S:P ratio, only active follicles in cross-section were counted. Inactive follicles at a level where only their ORS cell pegs were visible were not included in the count. Consequently, results on S:P ratio and the estimation of the percentage of active follicles should both reflect seasonal differences. Follicle activity percentages, however, did not show significant seasonal differences in this study.

Results on the changes in fibre diameter, length and S:P ratio suggest that selection for increased wool production in the Romney includes changes in fibre diameter and, through increased S:P ratio, also in follicle density. Fibre diameter increase appears to be the preferred mechanism. Follicle bulb size (diameter and area) was more strongly related to fibre diameter than to fibre length. If selection criteria do not place restrictions on fibre diameter, selection response appears to manifest itself through larger follicle dimensions. Thus, although fibre diameter and follicle density were reported to be negatively correlated in the Merino (Davies and McGuirk, 1987), both traits are able to increase concurrently, if the skin can accommodate the increase. It appears that breeds like the Merino, because of their special selection for extremely dense fleece, have reached the limit for accommodating follicles in the skin. This concurs with a concept by Moore et al. (1991) for the Merino, where follicles continue to form until some essential component of the skin is depleted. The skin has a developmental capacity to produce follicles in excess of the avialable initiation sites, determined by the size of the foetal pre-papilla cell population (Moore et al., 1991) Hence, every further improvement creates greater competition between follicles. There are problems in extrapolating the results from a breed like the Merino to other sheep breeds. Separate investigations for different breeds of sheep are necessary.

Energy metabolism

Hardly any glycogen was observed in the ORS during a period of low wool production (winter). The role and significance of glycogen for the hair growth cycle and energy requirements of the follicle bulb cells and growing hair fibre are doubtful, and need further investigation. Since the storage location appears to have no functional connection to the place where glycogen was supposed to be needed, its role might be altogether different. Storage of glycogen in the ORS has no apparent use for supplying energy to the follicle bulb, and an alternative role for this glycogen should be sought. The ORS may have a glycogen store for local usage as an energy source for the ORS cells during periods of accelerated growth (regeneration). A study by Cotsarelis et al. (1990) supports the idea that hair matrix cells of a new hair cycle in the mouse are derivates of a group of late replicating ORS cells below the sebaceous glands, emphasising the role of ORS in hair cycle events and the pluripotential nature of its cells.

Sites of activity of enzymes associated with aerobic glycolysis, such as succinate dehydrogenase (SDH) were sparse in the bulb tissue. There was no selection response of this to higher wool production. Therefore, anaerobic glucose utilization must also be necessary for energy requirements in the follicle, and may be of more importance for the follicle than it is for the skin (Chapman and Ward, 1979). This could be tested further by using skin perfusion preparations measuring metabolism of skin (Harris et al., 1989) or follicle cell culture (Ward and Harris, 1976; Philpott and Kealey, 1991) to measure oxygen uptake and lactate production of individual follicles.

Follicle morphology

A most noticeable selection response in fleeceweight sheep was the development of larger follicles, with an increased germinative tissue region in the bulb and a larger dermal papilla. Consequently, one mechanism controlling different levels of wool production suggested here is that fleeceweight selected sheep produce more wool from their larger follicles, accommodating a larger area of proliferating cells, producing at a proportionally similar rate to follicles of control sheep (Chapter 4).

Distribution of matrix cells to cortex and IRS

A mechanism responsible for changes in the distribution of bulb cells to either fibre cortex or IRS was observed to operate in sheep used in the present study. This mechanism appeared to be both under genetic and seasonal influence. It was directly related to flock

differences in wool production, in so far as it intensified the expression of genetic flock differences manifested by follicle bulb size.

FWT sheep had their germinative tissue areas growing proportionally with the area of the dermal papilla in summer. CLT sheep showed a disproportional enlargement of germinative tissue in summer, with a dermal papilla remaining similar in size to that measured during winter. Different genotypes thus exhibit different ratios of dermal papilla to germinative tissue. This, in turn, suggests a hypothesis concerning the distribution of cells to either the cortex or IRS. If the ratio of dermal papilla area to bulb area stays the same, with increased areas in both components, as observed in FWT sheep, most bulb matrix cells migrate to form the cortex. If, however, the ratio decreases, as observed in CLT sheep, an increased number of cells migrate into the IRS. This significance of dermal papilla size can be related to the concept of Nagorcka and Mooney (1982), based on a reaction-diffusion system of morphogens. Their theory states that the spatial inhomogeneities of three substances (X,Y in the bulb and Z in the dermal papilla) determine distributions and differentiation of cell types in the follicle. The morphogen of dermal origin, denoted Z, diffuses radially outwards through the follicle and is involved in cell differentiation. The product of the concentration of bulb morphogens and dermal papilla morphogen Z, under or above a certain threshold, then determines the differentiation of cells. To explain the morphological differences in the two lines of sheep, it is proposed that a higher concentration of morphogen Z is necessary for a cell to differentiate into a cortex cell, than is needed for that same cell to differentiate into an IRS cell. With a smaller papilla, shorter basement membrane surface area and a proportionally greater germinative tissue area, the morphogen Z would have to diffuse through a longer distance, and be more diluted before it reached the distal boundary of the germinative tissue. Consequently, more cells would differentiate into IRS cells, as observed in CLT sheep in this study. A diagram is given in Figure D.1 to illustrate these mechanisms.

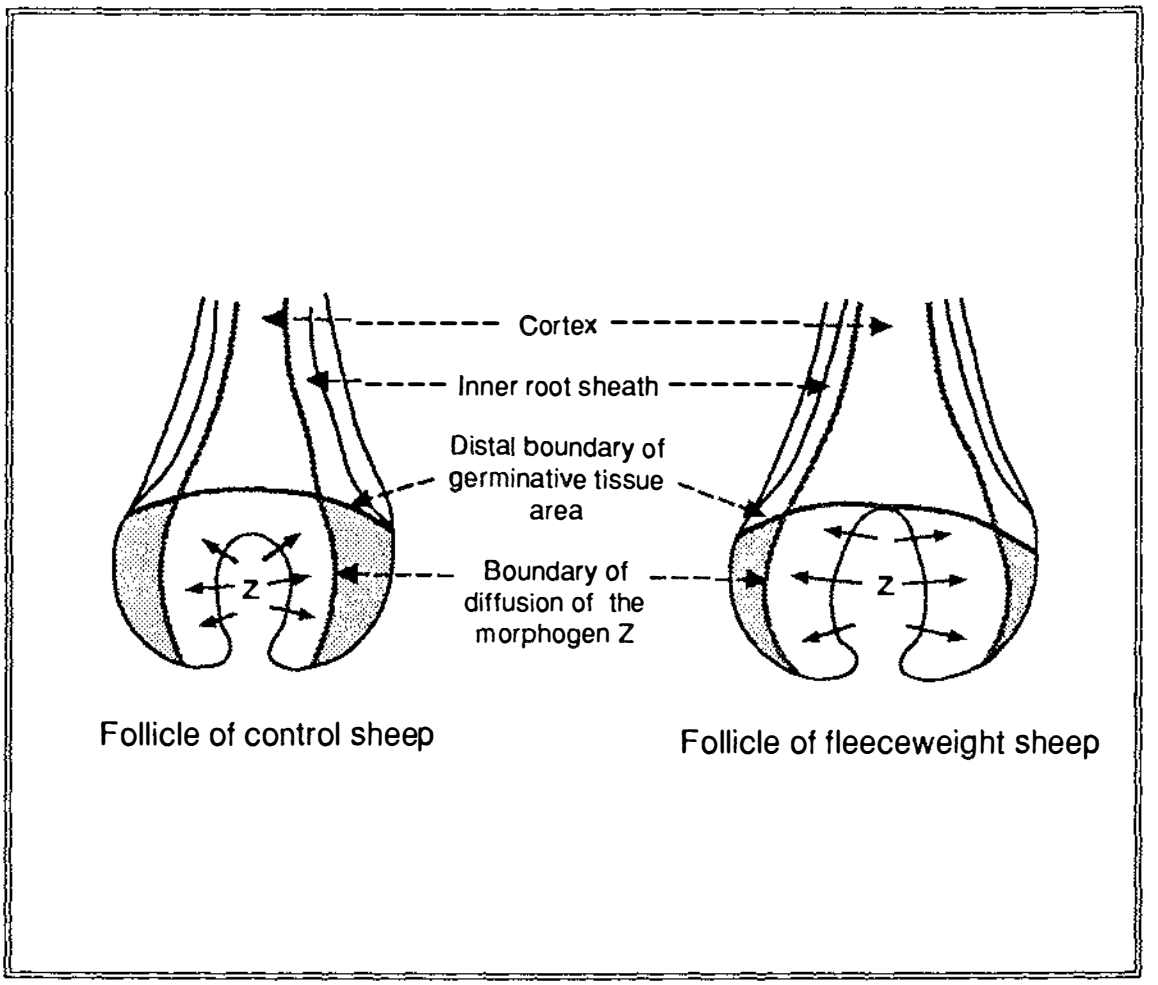

Figure D.1 **Schematic representation of different diffusion areas for morphogen Z (Nagorcka and Mooney, 1982), determining the differentiation of matrix cells into either cortex or IRS cells in CLT and FWT sheep** (shaded area: cells only under the influence of bulb morphogens, hence destined to differentiate into IRS cells; arrows: diffusion of dermal papilla morphogen Z into bulb matrix cells determining their differentiation into cortical cells)

Cell migration

The distribution of cells and cell lines in the bulb and to the different components of the follicle, including general migation patterns of bulb matrix cells, need further investigation. This study only offers an explanation based on two dimensional measurements. However, three-dimensional reconstruction of follicle bulbs is currently being investigated and will be applied to the same data used for this study. Thus, the relative magnitudes reported here for cell numbers and area proportions (Chapter 4) might change, but the overall concept derived from two-dimensional measurements is believed to remain valid.

A method is needed to label and identify bulb cells at their origin and after their

subsequent migration up the bulb. Only then can distinct cell lines and their fate be positively identified. Techniques have been reported using quail-chick chimeras to establish the origins of voluntary muscles in the avian head (Noden, 1983). Since sheep-goat chimeras are also possible (Fehilly et al., 1984), chimeric follicles could be produced by the localised injection of epithelial cells of a different genotype (goat) at precise positions within the basal bulb matrix cell layer around the dermal papilla. Histological detection of the subsequent position of these cells could elucidate cell migration patterns. Administration of cells could be achieved with separate follicles in culture medium in vitro (Ward and Harris, 1976; Green et al., 1986; Philpott et al, 1989; Philpott and Kealey, 1991). It would be a challenge to investigate whether these techniques are applicable to cell migration studies in the wool follicle.

Morphology of the dermal papilla

A larger papilla would be able to deliver more nutrients from its capillaries to the follicle, nourishing a greater mass of bulb tissue and making it easier for the follicle to compete for nutrients with other body tissues. Consequently, a relationship between the supply of blood to the follicle and the rate of wool growth should be determined. The genotypic model used in this study could be used to determine if FWT sheep can deliver nutrients at a greater rate to their follicles than can CLT sheep. Studies on skin blood flow of isolated skin patches have been established (Harris et al., 1988) to be used on genotypic extremes. No direct estimations of blood supply to individual follicles have yet been made. Neither has the density of blood capillaries around follicles, and especially within the dermal papilla been determined. Preliminary investigations (S.A. Holle, P.M. Harris and D.W. Dellow, unpublished) have developed a method of vascular corrosion casting of the skin capillary bed, to demonstrate patterns of blood supply to follicles.

Cell proliferation

The results on proliferation rates in the follicle were equivocal. At this stage it is not clear whether this uncertainty reflects a real biological variation or problems in measurement and analysis. The results on the density of proliferating cells in the germinative tissue area and on the relative difference in bulb area and number of proliferating cells, which account for the higher production in FWT sheep (Table 4.37) support the hypothesis that no rate changes are associated with increased production. However, results of the November samples, where rate changes were observed in FWT sheep, indicate that cell proliferation might be part of the selection response. Since this study did not investigate rate changes, additional work is required

to elucidate their relative importance to wool production in the same genotypic model. Changes in cell proliferation with time should be investigated, using either repeated injections of BrdU or a single injection followed by repeated sampling at different times after injection. The hourly increase in the number of labelled cells can then be estimated. Samples should be taken during different seasons to complement the data obtained in this study for November.

Follicle measurements proved to be only weakly related to fibre length growth rates. These rates could instead be associated with cell kinetic processes in the bulb. Where this the case, a change in proliferation rate may only occur if selection is targeted on fibre length growth rates and diameter changes were restricted. Mitotic density and rate processes in the follicle would be a fruitful subject for further investigation.

Table A.1 **Paraffin Wax Processing Schedule**

Process	Reagent	Time
Dehydration	70% ethyl alcohol	1 hour
	95% ethyl alcohol	1 hour
	100% ethyl alcohol	1 hour
	100% ethyl alcohol	1 hour
	100% ethyl alcohol	4 hours
	100% ethyl alcohol	4 hours
Clearing	Xylene	1 hour
	Xylene	1 hour
	Xylene	1 hour
Impregnation	Paraffin Wax 56^{0}C MP	4 hours
	Paraffin Wax 56^{0}C MP	2 hours

(1) Haematoxylin and Eosin (H & E)

Method:

1. Dewax and bring sections to water.
2. Stain in Mayer's Haemalum for 10 minutes.
3. Rinse in tap water.
4. Blue in Scott's tap water for 2 minutes.
5. Rinse in tap water.
6. Stain in 1% aqueous Eosin Y for 2 minutes.
7. Rinse rapidly in tap water.
8. Dehydrate, Clear in xylene and mount in D. P. X.

(2) Periodic Acid Schiff (PAS)

Method:

1. Dewax and bring sections to water.
2. Oxidise in 1% periodic acid for 8 minutes.
3. Wash in running tap water for 5 minutes.
4. Wash in three changes of distilled water.
5. React with Schiff's reagent for 10 minutes.
6. Wash in running tap water for 10 minutes.
7. Stain in Mayer's Haemalum for 5 minutes.
8. Rinse in tap water
9. Blue in Scott's tap water for 2 minutes.
10. Rinse in tap water.
11. Rinse in 10% and 95% alcohol.
12. Counterstain in saturated solution of Tartrazine in cellosolve (ethylene glycol monoethyl ether) for 2 minutes.
13. Differentiate and dehydrate in 2 changes of absolute alcohol.
14. Clear in xylene and mount in D. P. X.

Preparation of Schiff's Reagent:

To 400 ml of distilled water warmed to 37^{o}C add 2 g of parafuchsin and agitate until dissolved. Add 7.6 g of sodium metabisulphite and 2 ml of concentrated hydrochloric acid. Shake well for 10 minutes. Store in dark cupboard at room temperature overnight until solution becomes straw-coloured. Add 2 g of activated charcoal. Shake well and filter before use. Store in refrigerator.

(3) Succinate Dehydrogenase (Nachlas et al., 1957)

Special reagents:

(1) substrate

0.1 M phosphate buffer pH 7.6	7.5 ml
0.2 M sodium succinate (MW 270.15)	7.5 ml
Nitro blue tetrazolium[1] (1 mg/ml) in normal saline (made fresh each time)	15 ml

(2) phosphate buffer ph 7.6

potassium dihydrogen orthophosphate KH_2PO_4 (1.36 g/100ml)	10 ml
disodium hydrogen orthoposphate Na_2HPO_4 (1.78 g/100ml)	90 ml
distilled water	to 500 ml

(3) 10% formol saline (stock solution)

1 Nitro-BT: [2, 2'-di-p-nitrophenyl-5, 5'-diphenyl-3, 3'-(3,3'-dimethyloxy-4,4'-bephenylene) diterazoliumdichloride] (Novikoff, 1961)

(4) Succinate Dehydrogenase

Method:

1. substrate (1), incubate at 37°C for 20 min
2. distilled water, wash in 2 changes
3. dry sections to eliminate gas bubbles
4. 10% formalin, fix for 10 min
5. wash and mount in glycerine jelly

(5) Sacpic (Auber, 1952, modified)

Method:

1. Dewax and bring sections to water.
2. Stain in Celestin Blue for 10 minutes.
3. Rinse in tap water.
4. Stain in Mayer;s Haemalum for 10 minutes.
5. Rinse in tap water.
6. Blue in Scott's tap water for 2 minutes.
7. Rinse in tap water.
8. Rinse in 70% alcohol.
9. Stain in Winiwaters Safranin for 15 minutes.
10. Differentiate in two changes of 70% alcohol.
11. Rinse in two changes of 100% alcohol.
12. Rehydrate in two changes of 70% alcohol.
13. Rinse in tap water.
14. Rinse in distilled water.
15. Stain in Picro-Indigo Carmine for 2 minutes.
16. Differentiate in two changes of 70% alcohol.
17. Dehydrate in two changes of 100% alcohol.
18. Clear in xylene and mount in D. P. X.

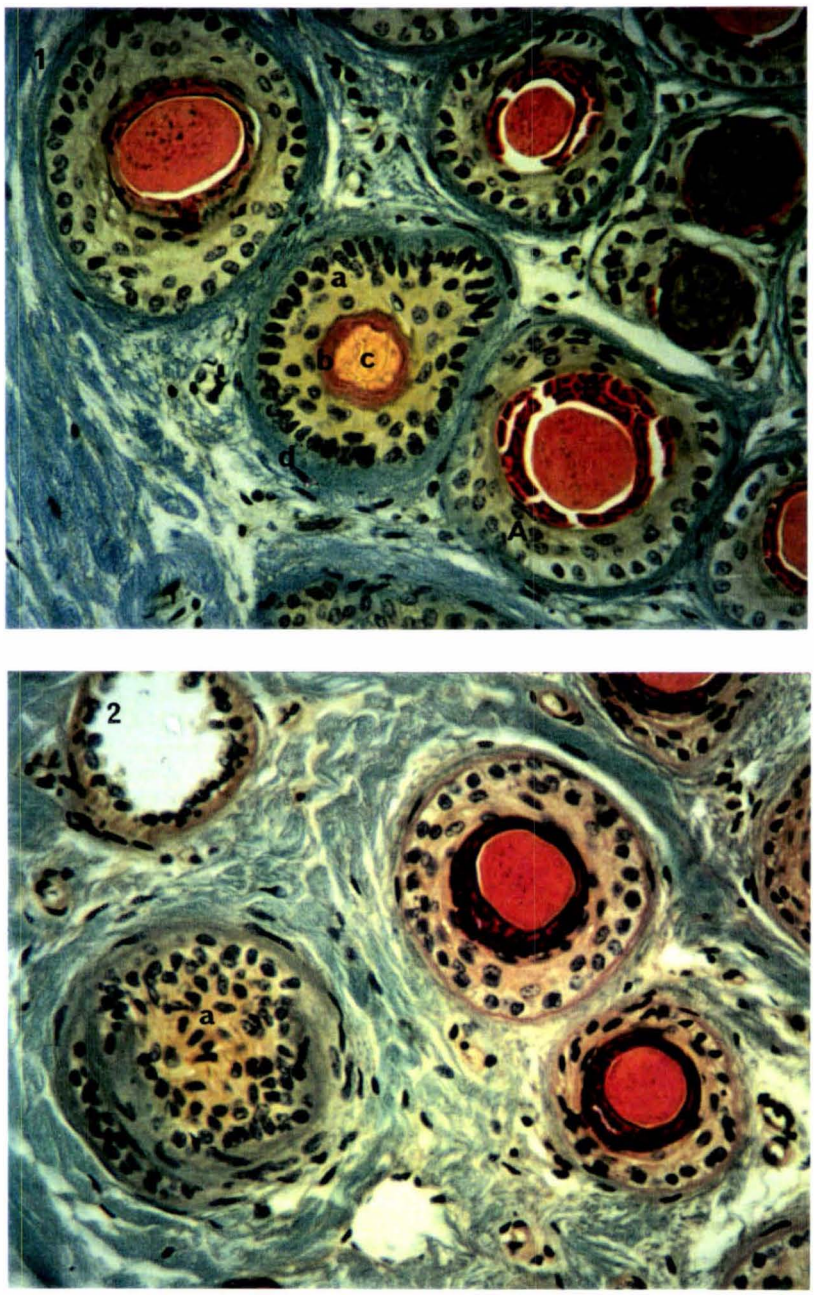

Figure A.1 **Cross-sections through several follicles at a superficial skin level (1) and a deep skin level (2), showing active follicles and one inactive follicle, stained with sacpic stain.** Magnification: 288x

(1) Some characteristic features of catagen and anagen follicles are: *Follicle in catagen showing* **(a)** ORS with ovoid, radially orientated cell nuclei; **(b)** IRS staining orange; **(c)** fibre cortex; IRS and fibre cortex showing irregular shape; **(d)** connective tissue sheath slightly enlarged. *Follicle in anagen showing* **(A)** round nuclei in ORS; **(B)** smooth IRS staining red; **(2)** At a level further proximal only ORS cells are visible in the catagen follicle: **(a)**

Table A.2 **Regression coefficients for the number of labelled cells per bulb section versus time**

regression equation: y = b + a * x				
sheep No	No of labelled cells versus time			
	b-intercept	a-slope	STD of a	R-sq %
1	37.703	2.4524	0.1252	67.2
2	46.401	2.1051	0.1748	43.3
3	29.479	2.3191	0.3567	25.9
4	29.107	1.5769	0.2562	30.1
5	38.036	2.4261	0.4745	21.8
6	46.389	0.9152	0.4354	02.9

Table A.3 **Mean values and SEM for the number of labelled cells per bulb section**

sheep No	1		2		3		4		5		6	
time/h	NO	SEM	NO	SEM	NO	SEM	NO	SEM	NO	SEM	NO	SEM
0.05	-	-	-	-	-	-	27.4	7.79	-	-	25.5	1.17
0.25	28.9	1.59	30.8	1.45	30.8	2.82	19.5	2.63	42.6	4.76	-	-
0.50	39.1	1.40	42.0	1.98	35.2	2.00	-	-	31.6	3.12	44.3	1.79
0.75	42.8	1.62	50.2	2.01	31.5	2.08	32.6	2.93	-	-	76.6	2.62
1	46.7	2.11	54.0	1.74	30.7	1.68	30.7	2.30	46.9	2.87	37.8	3.18
2	45.6	2.62	55.9	2.32	34.8	2.50	35.3	2.21	36.7	4.86	30.9	1.74
4	47.9	1.30	66.4	3.56	28.9	2.03	-	-	46.8	2.31	42.5	1.48
6	53.8	2.81	68.2	3.81	43.9	2.87	29.5	0.50	49.3	3.71	52.3	1.77
8	53.0	2.13	63.8	3.08	51.8	3.75	41.8	1.83	58.4	6.81	56.2	1.43
10	49.4	2.04	54.8	2.39	-	-	44.6	3.53	66.5	4.98	-	-
24	99.2	5.19	101.13	6.39	-	-	-	-	-	-	-	-

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