The short term effects of stress hormones on cell division rate in wool follicles.

A thesis

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by

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Abstract.

A local intradermal technique using colchicine to estimate cell division rate in wool follicles has been refined and used throughout this thesis. The statistical methods used to analyse data obtained with this method are described and discussed.

An initial investigation into possible circadian rhythms in the rate of cell division, revealed erratic fluctuations of up to six fold, rather than a sinusoidal rhythm as seen in earlier work. It was proposed that this discrepancy might be attributed to the effects of stress hormones released in response to handling of the animals. Literature evidence suggested that wool growth is affected by cortisol released in response to stress, and that plasma cortisol levels exhibit circadian rhythms. It was hypothesized that cortisol might cause both sinusoidal and erratic fluctuations in cell proliferation. However, extensive investigations in sheep adrenalectomised using a new technique, revealed that neither intradermal injections of cortisol, nor imposed changes in plasma cortisol level affected cell division within less than one day. This claim was further supported by absence of a response of *in vitro* DNA synthesis in skin strips incubated in the presence of cortisol, with respect to control samples.

In contrast, *in vitro* DNA synthesis was perturbed by the presence of catecholamines in the medium. Cell division rates *in vivo* were similarly reduced by local injections of adrenaline and noradrenaline. Endogenous secretion of noradrenaline was also shown to reduce cell division with respect to sites that had been surgically sympathectomised. Chemical sympathectomy was also attempted on a limited number of animals, and yielded similar yet less convincing results.

Finally, the presence of a circadian rhythm in cell division was re-assessed using a less stressful sampling protocol. Once again, this revealed no sinusoidal rhythm, but examination of available observations suggested that rapid changes in cell division were commonly observed in the early morning, in housed animals which are accustomed to morning feeding and cleaning procedures. The implications of these findings are of great significance to research into the influence of physiological changes on wool production, and suggest that experiments should be conducted under controlled environmental conditions, with a minimum of stress imposed on the animals.

Declaration.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

David Scobie.

Acknowledgements.

To acknowledge those who have helped in my journey through this postgraduate training I would like to dedicate the following poem. In order of appearance in the verses, the names refer to: Dr. P. I. Hynd, Prof. B. P. Setchell, Dr. A. Foldes (CSIRO, Blacktown, NSW), Elke Henning (Flinders University, S. A.), Dr. T. Kuchel (Flinders University, S. A.), Ron Newman (CSIRO, Blacktown, NSW), David Baird (MAFTechnology, Lincoln, Christchurch.), David Saville (MAFTechnology, Lincoln, Christchurch.), Andrew Dunbar, Bronwyn Everett, Selena Emery, Jenny Bennett (plus Rowan Bennett), Dr. N. Edwards, Janelle Hocking Edwards, "The Mullets" (Andrew, Tim, Paul, Mark and Anne), Andrew Peter Duncan "Spike" Lord, Preethi Perera, Jenny Prosser, Rex Connolly, Tony Weatherly, the Spavens, the Scobie families, Susie Laube, Jim Zupp and the Australian Wool Corporation.

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at the flick of my shearing wrist,
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a debt of thanks I am owin'
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and often supplied me with credit,
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Susie you more than those listed above,
gave me the strength to help finish it.

If your name doesn't appear on this list,
please don't you feel unhappy.
My poetical skills meant it was missed,
after all, what the heck rhymes with Zuppy.

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for supplying my scholarship,
in the worlds best wool-growing nation,
thanks for the ticket to the trip.

Chapter One.

Review of Literature.

1.1. Morphology and function of skin and follicles.

The initial section of the following chapter provides a concise description of the histomorphology and development of wool-growing follicles located within the skin of sheep. Fibre growth and environmental factors which affect the rate of wool growth from the follicles are also addressed. The second section of this first chapter deals with the effects of more specific factors such as endocrine and neuroendocrine activity on the rate of wool growth. A variety of techniques that have brought us to our present level of understanding of the processes of wool growth are reviewed in the third and final section of the chapter. The literature reviewed in this manner forms the basis for the experiments reported in subsequent chapters. Definitions which have been adhered to throughout the following text are listed below.

1.1.2. Skin.

The dictionary definition of the word 'organ' is as follows; A fully differentiated structural and functional unit. The skin of an animal is not commonly thought of as an organ, yet it remains the largest organ of the vertebrate body. The skin is of "structural" importance in retaining the position of other organs such as those in the visceral cavity with the assistance of muscle, fat and connective tissue layers. As a "functional unit" the skin serves a number of life-preserving roles, and many of these are interrelated. Like some other organs, to conduct certain activities, the skin must not be "fully differentiated". Certain progenitor cell lines within the skin remain undifferentiated and continuously-dividing throughout the life of the animal. The functional tissues of this organ are produced by differentiation of daughter cells of this division. These cell lines, and the coordination and control of some functions of this organ are described in the following sections and form the subject of interest in this study.

1.1.3. Skin: Morphology and Proliferation.

The skin of vertebrate animals is made up of three major layers. From the exterior these are, in order; the epidermis, the dermis and the underlying subcutaneous fat. At its junction with the dermis, the epidermis exists as a basal stem cell layer in which cell division occurs. Some of

the daughter cells move toward the exterior after leaving the basal layer and progressively become filled with structural proteins called keratins. The keratin-filled cells desiccate and become harder as they approach the surface, and flatten out over the the cells below. Finally, the cells are lost from the surface by wear or by sloughing. The dermis on the other hand consists of amorphous connective tissue interspersed with single cells such as fibroblast cells, mast cells and macrophages, and groups of cells forming tissues, for example nerves and blood vessels.

1.1.4. Skin: Modification to form additional structures.

Interspersed in the skin, or in specialised regions, additional structures develop from associations of both the dermis and the epidermis in the foetus and newborn, and are continually renewed in the adult by a similar process to that which occurs in the epidermis itself. These have been recently reviewed by Chapman (1986), Hashimoto *et al.* (1986) and also by Quay (1986). Only a brief description of function and form of these structures will be given here, to demonstrate the diversity of structures developed as part of the skin for a variety of purposes.

Glands are a simple example of a regional modification of the skin and consist of epidermal cells lining a down-growth or pit into the dermal layer. These are either exocrine glands which secrete fluids, or holocrine glands that slough off entire cells by an exfoliative process similar to that which occurs in the epidermis itself. Sweat glands are exocrine glands which provide a means of evaporative cooling whilst simultaneously controlling water loss. Sebaceous glands are an example of holocrine glands, and provide lipid secretions which waterproof and protect the epidermis and other derived structures from the environment. These glands also provide a vehicle for various sex secretions, and scent markers or pheromones (Quay, 1986) and a route for excretion of lipid-soluble wastes.

Another group of structures are outgrowths of epidermal tissue, principally composed of keratin proteins. Universally they develop from intrusions of the epidermis into the dermal layers; examples include the nailbed and the hair follicle (Chapman, 1986). These structures

include; "tools" such as claws, hooves, horns, flight feathers and nails; and "insulating materials" like down feathers, hair, fur and wool. The first group of these serve mainly protective or utility functions and often incorporate formative elements of the underlying bones, fat deposits, connective tissues, muscle and cartilage e.g. the bovine horn and hoof (Chapman, 1986).

The latter group, the insulating materials, are the structures of interest in the present work and are labelled in general terms, the pelage. These structures aid in thermoregulation of the animal and also protect the underlying epidermis from harmful ultraviolet radiation, abrasion, wetting and other environmental hazards. In serving these purposes, the pelage sustains much wear and tear and as with the epidermis itself, it is necessary to replace the elements of the pelage on a regular basis. The wool follicle of the sheep *(Ovis aries)* and the continual growth of the wool fibre from this follicle are the principal subjects of this thesis, and the main aim of the work is to understand the regulation of fibre production. An understanding of the histology or histomorphology of the tissue is essential in any study of this nature. This fact is highlighted by the comment: "There is no real difference between structure and function; they are the two sides of the same coin. If structure does not tell us anything about function, it means we have not looked at it correctly". This quote originated from Szent-Györgyi, (1951) and has since been reiterated by Montagna (1956) as a foreword to the book, "The Structure and Function of Skin", and also by Rothman (1958) in his introduction to the book "The Biology of Hair Growth". This quote is very relevant to the present work, and it is pertinent here to briefly describe the development and form of wool follicles in more detail, as background to the main text.

1.1.4.1. Wool Follicle Development.

Hardy and Lyne (1956 a and b), Fraser and Short (1960) and Ryder and Stephenson (1968) provide detailed descriptions of the development of a follicle and also the follicle population of the skin as a whole.

Wool and hair follicles begin development as a simple swelling or bulge of epidermal cells at the junction between the dermis and the epidermis. An aggregation of dermal cells forms in close proximity to this overlying bulge of epidermal tissue. This bulge develops further by the epidermal tissue intruding into the underlying dermis. The base of the anlage begins to flatten over the aggregation of dermal cells and later envelops these cells, which eventually become the dermal papilla. Concurrently two types of accessory structure begin to develop at the sides of the forming follicle and eventually become the sebaceous and sudoriferous glands (or holocrine and exocrine sweat glands respectively). A branched smooth muscle also develops along with the follicle to become the arrector pili muscle.

After the follicle has assumed its shape, the intruding epidermal tissues hollow out to form a tube by cell death and degradation. The tube extends from just above the dermal papilla to just below the surface of the epidermis. Within this canal a cone of tissue forms above the papilla, and eventually becomes the outer layer of the inner root sheath. This layer is surrounded entirely by cells of epidermal origin known as the outer root sheath, which delineates the follicle and separates it from the surrounding dermal tissue. Concentrically within the outer root sheath, three layers of the inner root sheath form. The outer layer is referred to as Huxley's layer, which surrounds Henle's layer, which encloses the inner root sheath cuticle.

At about the same time as the tube forms above the papilla, the amorphous cells surrounding the dermal papilla begin to divide and daughter cells migrate up through the tube or hair canal to eventually form a fibre which breaks through the surface.

While the follicles have been developing in the above manner, so has the foetus and its increase in size gives rise to the development of further follicles so that a dense pelage is present at birth, to protect the neonate. Follicle development expands to cover the body surface, firstly to form individual follicles like the central primary follicle described above, followed by the development of lateral primaries and finally secondary original and secondary derived follicles. Secondary follicles resemble the primaries except for the fact that they have much diminished sebaceous glands and no sudoriferous gland or arrector pili muscle.

An important feature which accompanies wool follicle development is the growth of a nerve and capillary network in the vicinity the follicle. It is interesting to note that although the development of the follicle itself has received much attention, it seems that the development of these structures within sheep skin, as a whole organ, have been neglected. The literature contains scant information on the final histomorphology (Ryder, 1955; Lyne and Hollis, 1968), and no information on the development of these networks. This gap in our knowledge of the development of sheep skin persists, despite the importance which some authors have attributed to nerve and blood vessel function and their interaction in sheep skin and wool production (Ferguson, 1949; Reklewska, 1975; Cunningham *et al.*, 1979; Wallace, 1979a; Williams and Willis, 1987).

1.1.4.2. Fibre growth.

1.1.4.2a. The germinative region of the follicle bulb.

The base of the follicle or 'follicle bulb' is filled with amorphous cells, which originate from epidermal cells during the process described in the previous section, and continuously divide by mitosis to produce a fibre. These cells are referred to as the matrix, germinative tissue or proliferative region interchangeably by numerous authors. In the past there has been some debate over the limits to this germinative tissue. Some of the earliest work was conducted using follicles from mouse skin by Auber (1950), who proposed a 'critical line' in the bulb, below which all division occurs. Bullough and Laurence (1958) on the other hand described the mitotic tissue as a "ringlike matrix which surrounds the dermal papilla."

Various other workers have provided additional descriptions of the matrix of wool follicles. Short *et al.* (1965) used a subjective means of assessing the outer limits of the mitotic tissue. The criteria used were: " the positions of distal mitotic nuclei, the change in cell size and the level at which differentiation of the inner root sheath and cuticle cells became apparent." Fraser (1965) used the term "germinative tissue" to describe a combination of both mitotic and differentiating cell zones. The mitotic cell zone occupied roughly 65% of the germinative tissue.

Finally, Epstein and Maibach (1969) using human hair bulbs provided the following figure (Figure 1.1) to show the limits of the germinative cell population.

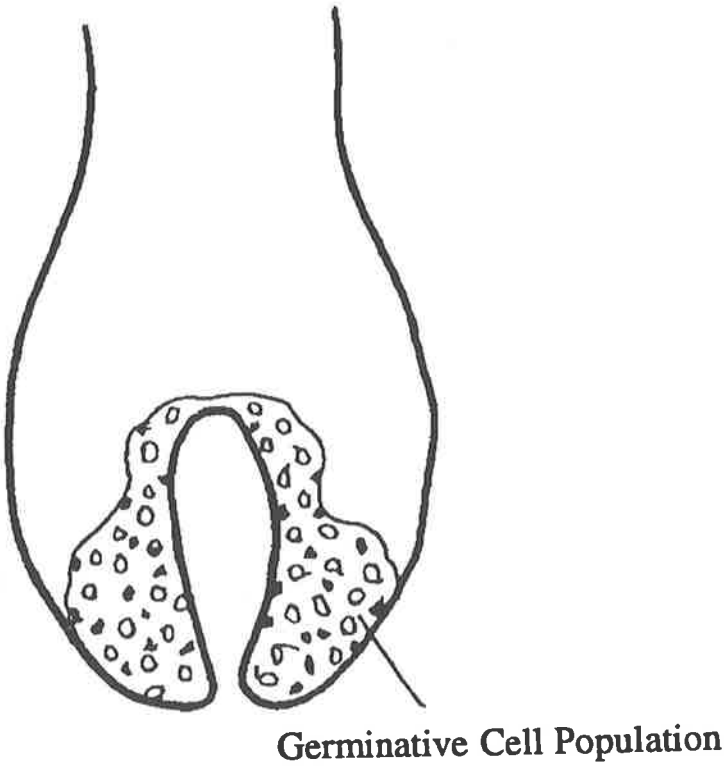


Figure 1.1. The limits of the germinative cell population. Taken from Epstein and Maibach (1969)

It is most important to note the point made by Epstein and Maibach (1969) to accompany Figure 1.1, viz: " Schematic representation of the germinal centre for the hair bulb. **Its confines are not so readily apparent in reality.**" The rate of cell division in the bulb is of much greater importance to the present work than the decision where to draw a boundary around the dividing cells, and the limits of the region are mentioned here to define the tissue which is studied at length in the experiments to follow.

N.B. The present author has observed numerous histological sections of wool follicles after the arrest of cell division by colchicine. After relatively short periods of arrest (two hours) wool follicles resemble Figure 1.1, although the proliferative region appears more elliptical in shape. With much longer periods of colchicine treatment (six hours), arrested mitoses can be found at sites described by Auber (1950).

1.1.4.2b. Wool fibre formation.

For the purposes of the present report, wool growth can be considered as a four stage process:

Cell Division: mitotic events in the follicle bulb.

Migration: departure of some daughter cells of the mitotic division from the bulb.

Differentiation: changes in size and shape of the cells after leaving the bulb.

Keratinisation: formation and hardening of keratin proteins within the cells.

Figure 1.2 provides a clear and detailed diagrammatic representation of a longitudinal section through a wool follicle. This illustration was modified from Orwin (1976) and specifically represents the follicle of a Romney sheep. In general all wool follicles assume this form and only the approximate dimensions differ. The associated regions for cell division, differentiation and keratinisation are labelled respectively as: mitotic zone of bulb, elongation zone and keratogenous zone on Figure 1.2. The diagram (Figure 1.2) neatly summarises the morphology of the wool follicle and some of the processes which occur in the different regions.

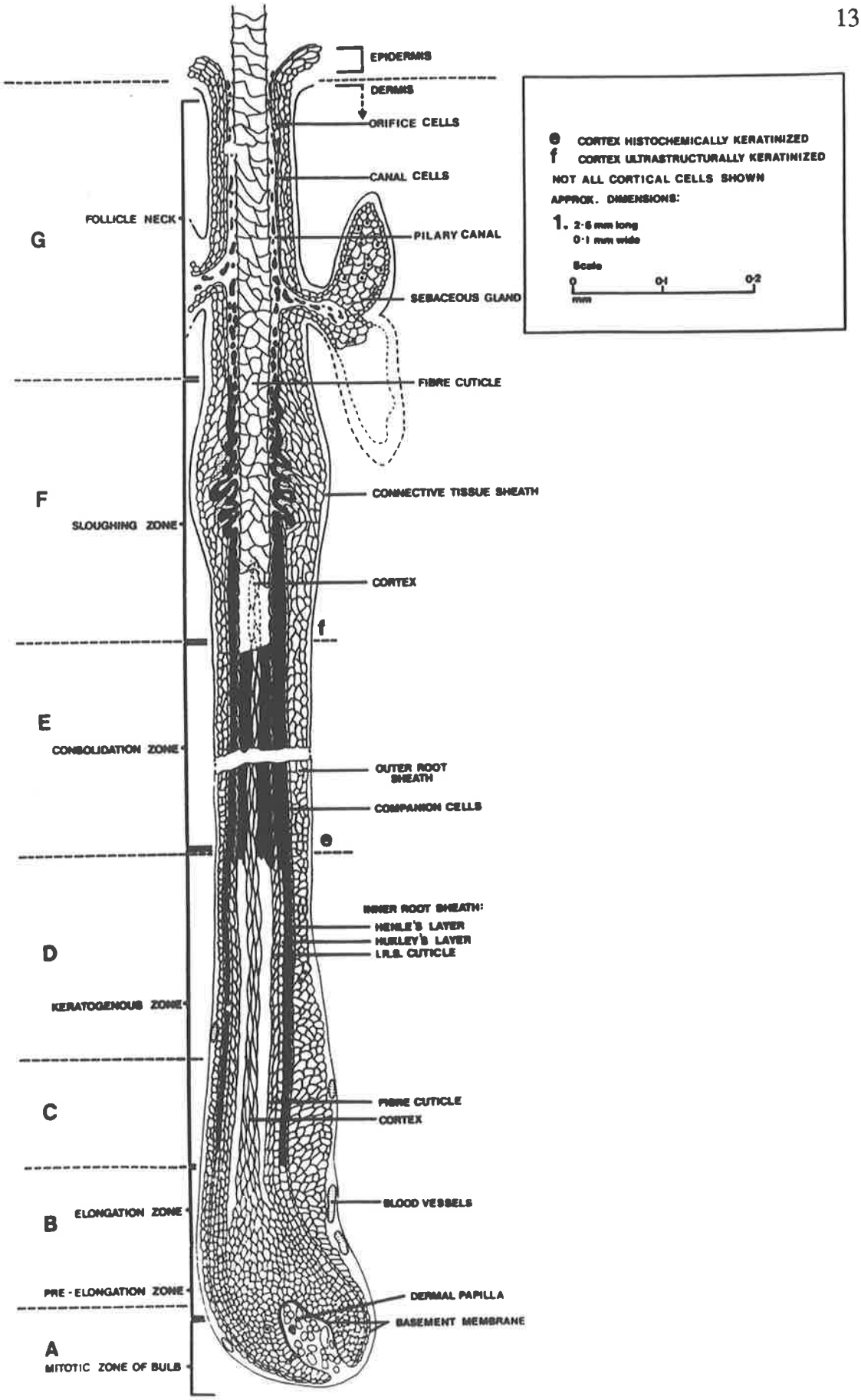


Figure 1.2. Diagrammatic representation of a wool follicle sectioned longitudinally. Modified from Orwin (1976). This figure demonstrates the morphology of the wool follicle. The processes which occur in many of the zones outlined on the figure are further described in the text.

1.1.4.2b(i). *Cell Division.*

Cell proliferation rate in the follicle bulb is a function of the total number of cells in the matrix and the rate at which that number of cells is renewed, or the turnover time of those cells (Wilson and Short 1979). Turnover time is defined as; the number of hours required for replacement of the entire population of cells in the follicle bulb. Some estimates of the rate of cell proliferation rate are provided in the following table (Table 1.1)

Table 1.1. The average number of cells per bulb and turnover time of bulb cells, as determined by various authors.

Cell turnover time (hours)	Number of cells per average bulb.	Authors
19 to 42	731 to 816	Short *et al.* (1965)
18 to 59	438 to 771	Fraser (1965)
25 to 47	493 to 1620	Hynd (1989a)

By pulse-labelling with [^{3}**H**]thymidine and subsequently counting the percentage of labelled mitoses, Downes *et al.* (1966) gave an estimate of a turnover time of 21 hours for the cells in the follicle bulb. All the examples in Table 1.1 were calculated using cell division arrest techniques which will be described later (Section 1.3.2(b).). From this information, it is evident that the entire population of cells in every follicle are renewed every one to three days. Considering the numbers of cells present in the bulb, of the Merino ewe studied by Short *et al.* (1965) (see Table 1.1) and the number of follicles per square millimetre of skin (of the order of 40 follicles per square millimetre for similar sheep (Hogan *et al.*, 1979)), an estimate of the maximum rate of cell proliferation per square millimetre of skin is in the vicinity of 29 000 to 32 000 cells per day (= # follicles x # cells / follicle). Using other techniques (described later in Section 1.3.2(a).), Ward and Harris (1976) have shown that roughly 88% of the DNA synthetic activity of the entire skin occurs in the follicle bulbs. From these observations, it is evident that the growth of wool involves a considerable commitment of energy and substrate resources.

The major point of interest with respect to the present work is the relationship between cell division rate or turnover time and the rate of wool growth (Schinckel 1961, 1962; Fraser 1965; Short *et al.*, 1965; Wilson and Short 1979; Hynd *et al.*, 1986; Scobie and Hynd, 1987; Williams and Winston, 1987; Hynd, 1989a). In general, these various authors have demonstrated that as the rate of cell division per follicle increases, so too the rate of wool production increases. This has either been shown to be related to the difference in genetic potential between animals or to the level of nutrition within the one animal.

The recent work of Hynd (1989a) provides an example of the effect of nutrition, where ten wethers were first fed a low protein ration, followed by a high protein ration. The change in plane of nutrition caused a 33% increase in wool fibre production in these animals; this was reflected in a 35% increase in the rate of proliferation of cells in the bulb and an increase of 30% in bulb area. During the same experiment, the number of mitoses arrested per bulb per hour was greater in high-producing Corriedale and Strongwool Merinos than in the relatively low-producing fine woolled Merino at both levels of nutrition.

Williams and Winston (1987) produced further evidence which demonstrated genetic differences in the rate of cell division, using animals which had been genetically selected for (Fleece Plus) and against (Fleece Minus) fleece production. These workers produced evidence which suggested that there was no difference in the average mitotic density (the rate of cell division per unit volume of germinative region) in the follicles of these sheep. Although it is not possible to determine the rate of cell proliferation per follicle directly from their data, some crude manipulation of their results is possible. The Fleece Plus group exhibited a larger germinative cell population per follicle (defined by these authors as the area of mitotically active cells) at both levels of nutrition, yet the mitotic density was only slightly lower in the Fleece Minus group. Evidently then, the Fleece Plus animals possessed larger follicles (and correspondingly larger fibres), yet had the same mitotic density, and therefore exhibited a greater number of mitotic cells per individual follicle. Those sheep with a genetically superior rate of wool production exhibit a greater rate of cell proliferation per follicle, but not per unit volume of follicle bulb.

1.1.4.2b(ii). *Migration.*

After cell division has occurred, the cells begin to migrate out of the bulb. During migration up the follicle shaft, the cells increase in size and differentiate to form the tissues described in Section 1.1.4.2b. From work conducted by Short *et al.* (1965), Wilson and Short (1979) and Hynd (1989a) it is apparent that the proportion of cells which migrate to form the fibre is low (9 to 20% of the entire population of migrating cells). Hynd (1989b) has shown that phenotype has a major influence on this proportion, and that it may be independent of nutrition.

1.1.4.2b(iii). *Differentiation and Keratinisation.*

As the cells migrate up the shaft of the follicle, they differentiate into the various tissues outlined above. The cells of the cortex elongate to form spindle-shaped cells and become filled with proteins which are known as keratins. The cortex becomes surrounded by cells which form the cuticle; these cells then flatten out and overlap at the edges in a tile-like fashion, and also fill with keratins. The inner root sheath cells encase the cuticle and migrate in the same manner (at a different rate (Chapman *et al.*, 1980)), without forming keratins, so that as the newly-formed fibre nears the skin surface these cells break down to leave the fibre which emerges from the skin. The fate of the inner root sheath cells is not completely determined; however, it is believed that some of the constituents are reabsorbed and the remainder is sloughed off and lost to the environment (Auber, 1950; Gemmell and Chapman, 1971).

In summary, the epidermally-derived cells in the base of the follicle or "the follicle bulb", continuously divide for prolonged periods in the skin of modern sheep breeds. The daughter cells produced by this division either remain in the bulb, or contribute to the fibre by migrating out of the bulb and up through the follicle shaft. As the cells migrate they either develop into the inner root sheath, or the fibre. The cells of the fibre elongate and become filled with keratin proteins as they migrate toward the skin surface, and these proteins harden or keratinise to form the fibre which emerges from the skin surface. The fibre is made up of several types of cells; the main bulk of cells form the core of the tissue and are referred to as the cortex.

Fibre formation continues in this fashion throughout the life of the animal, but the rate of growth and characteristics of the fibre may fluctuate. Since wool production is economically very important, factors which affect wool growth assume economic significance along with interest of a purely biological nature. Some of the many factors which affect rates of wool growth are reviewed in Section 1.2. below.

1.1.5. Skin and homeostasis.

The action of the sweat glands and the pelage, and cooperation between the skin and the rest of the body make the integument an important homeostatic organ of the mammalian body. Thermoregulation and to a lesser extent osmoregulation, are two very important homeostatic functions of the skin, and since they are interdependent and often antagonistic goals, a number of complex mechanisms are employed to achieve them. Many of the mechanisms are not fully understood individually, and less so the integration of them all. A recent review is provided by Cena *et al.* (1986). It may be of some significance that Brook and Short (1960) have reported the absence of sweat glands in a mutant strain of Merinos. After comparing these animals with normal Merinos in a hot environment when both groups had been shorn, it was suggested that sweat glands may have a reduced functional significance in sheep of this breed, although the authors made the additional comment that sweating may differ in woolled and shorn sheep.

Bottomley (1979), in a review of the effects of climate on wool growth, concluded that hot conditions are unlikely to reduce wool production directly, based on evidence produced by Thwaites (1968) and Cartwright and Thwaites (1976), who showed that all of the depression in wool growth under such conditions could be attributed to a reduction in food intake. Bottomley (1979) also pointed out that wool is an effective insulator against heat on well fleeced animals.

In contrast, cold environmental temperatures do appear to influence wool growth rates under certain conditions (Bottomley, 1979). Once again, in well fleeced sheep, wool growth is apparently not directly influenced by low temperatures. However, Bottomley (1979) presented a number of examples in which a localised reduction in wool growth rate was produced by

exposure of small areas of skin to cold by; shearing, close clipping, artificial wind and surgically implanted heat exchange chambers.

Wheeler *et al.* (1977) had previously shown that when the entire animal was shorn, fleece production actually increased. This experiment was conducted using groups of sheep which were either rugged or not rugged after shearing. The increase in wool output was attributed to differences in feed intake between the two groups.

Wodzicka-Tomaszewska and Bigham (1968) and Bigham (1974) provided evidence to suggest that results obtained using the harvest of wool from small patches to index whole body wool growth must be viewed with some caution. Frequent harvesting of the tattooed patches (discussed in Section 1.3.1 below) exposes the skin to the environment which would not normally be the case, and may exaggerate the effects of the season by cooling a small area of skin.

The salient features from this section are that:

1) environmental temperature *per se* does not influence wool growth of the entire animal.
2) localised temperature differences on the one animal can reduce wool fibre production at a local level.

1.1.6. Skin and sensory perception.

As skin is a homeostatic organ, sensing perturbations in and making the appropriate response to an ever-changing environment, (both internal and external) is an important function, and crucial to the survival of the mammal. The skin of an animal is generally richly-innervated and hosts a variety of sense organs for receiving various stimuli, including; thermoreceptors (Breipohl, 1986), mechanoreceptors (Malinovský, 1986), and chemoreceptors (Reutter, 1986). Skin is thus the single most important organ involved in receiving a range of environmental stimuli, such as touch and taste. Structures derived from the skin and underlying tissues are incorporated into the organs involved in the sensory perception of sight, sound and smell. The nervous function of the skin thus deserves consideration as it may be important in mediating

changes in other activities such as the growth of the pelage. This appears to be the case in the growth of wool and Section 1.2. below deals with this in greater detail.

1.1.6.1. Response to perceived changes.

The skin must be able to respond quickly to some stimuli to remain functional and intact. For example, wounding of the skin produces a rapid response in the migration of white blood cells to ward off invading pathogens while epidermal and dermal cells tend to the business of repairing the wound. The response to wounding, and indeed the way in which the skin is signalled to cease developing when it attains a given size and shape has long been the subject of much debate and research. To date the problem has not yet been completely resolved and it is beyond the scope of the present report, however Bereiter-Hahn (1986) provides a recent review.

The response of wool growth to environmental stressors is an important facet of this thesis and may require a relatively rapid reaction (acute) or a slower response (chronic) of the entire body of the animal. The response may involve a reaction to the degree where longer term functions of the body must be sacrificed in an attempt to ensure the short term survival of the animal. In such life or death situations, wool growth is often sacrificed; examples of these are provided in a review by Donald (1979), and involve stressors such as microbial infections or severe cases of blowfly strike. Under some circumstances, wool growth seems to be privileged, in that wool growth continues in the face of decreased food intake and liveweight (Foldes *et al.*, 1984. The steroid; cortisol (chronic), and the catecholamines; adrenaline and noradrenaline (acute) are the major hormones released in response to various stressors in sheep. The effect of these stress hormones on wool growth are reviewed in Section 1.2 below.

1.1.6.2. Seasonal Pelage changes.

The appropriate response to a given stimulus does not always involve a rapid response as described above for wound healing or stress responses. This is especially relevant to the skin, as a given short term change in the environment may be part of an even greater long term

change. Seasonal changes in the environment which bring about seasonal changes in the pelage of animals can be regarded as long term changes e.g. moulting or shedding, especially in those which live in temperate and cold climates. A good example of seasonal shedding is that evolved by the varying arctic hare or snowshoe rabbit (*Lepus americanus*), (Keogh,1969). This animal not only demonstrates a seasonal moulting pattern, but the pelage changes colour from agouti to white, to provide camouflage in the prevailing topographical conditions.

1.1.6.3. Seasonal phenomena in sheep.

In an article in "National Geographic" magazine (Hyde and Wolinsky, 1988) it was estimated that sheep were domesticated approximately twelve thousand years ago and shepherded to provide meat and skins. The authors suggested that once it was realised the animal was as valuable alive as it was dead, it gradually became used for milk production and finally for wool fibre production. It was also suggested that since these shepherding practices began, in all but a few of the "more primitive" breeds (e.g. the Soay and the Wiltshire Horn), the annual shedding of the fleece has been suppressed. This is presumed to be the result of many generations of selection for the growth of greater quantities of wool and the introduction of techniques for regularly clipping the fleece from the animal.

Pelage growth is not the only bodily phenomenon that is regulated by the changes in the season. Probably the best documented of other phenomena is that of seasonal breeding, where animals in temperate environments have evolved mechanisms to perceive changes in the daylength. The useful purpose of this is to ensure that offspring will be born at a time when weather conditions and food supply are at the optimum for survival. Although many sheep breeds appear to have lost the ability to shed their fleece seasonally, as mentioned above, many remain seasonal with respect to reproductive characteristics. A recent review of seasonal breeding in sheep (Kennaway *et al.*, 1987), describes the process in more detail and it is mentioned here purely to show that despite thousands of years of selection, sheep remain seasonal in some important respects.

1.1.6.4. Seasonal variation in wool growth.

Seasonal variation in wool growth rate without shedding is evident in both the amount and the characteristics of the wool fibre produced, but the extent of the changes with respect to season is variable. The "primitive breeds" mentioned above lie at one extreme and exhibit seasonal shedding. This has been shown to be regulated primarily by daylength in the Wiltshire horn (Slee 1965; Ryder, 1969, 1971, 1973 a and b) and the Soay (Lincoln *et al.*, 1980), although nutritional stress can modify the annual shedding of the fleece in the Wiltshire horn (Slee, 1965).

In a review on "Climate Physiology of Sheep", Hutchinson and Wodzicka-Tomaszewska (1961) presented evidence that seasonal changes in the rate of wool growth in wool growing sheep were related to the seasonal availability of pasture. In support of this, the rate of wool growth is highly dependent on the level of nutrition (reviewed by Allden, 1979), and severe nutritional stress has been shown to cause the shedding of fibres from up to forty percent of the follicles of Merino sheep (Lyne, 1964).

Although other breeds do not exhibit seasonal shedding of the entire fleece, they often exhibit an annual rhythm in wool growth rate, and under conditions of constant nutrition these seasonal cycles in the rate of wool growth were not completely suppressed (Ferguson *et al.*, 1949; Coop 1953; Coop and Hart 1953; Wodzicka, 1960a, 1960b). A collection of examples of experiments, in which intake was controlled and natural or near natural photoperiod imposed, is provided in a review by Nagorcka (1979). Even the Merino breed (which could be considered to lie at the other extreme to the shedding breeds) has been shown to exhibit seasonal changes in wool growth under such conditions (Ferguson *et al.*, 1949; Bennett *et al.*, 1962; Hutchinson, 1962; Williams, 1964; Doney, 1966).

When considering these experiments, it is important to keep in mind the work of Wodzicka-Tomaszewska and Bigham (1968) and Bigham (1974) (discussed in Section 1.1.5. above), since much of the information was obtained using the tattooed patch technique.

Bennett *et al.* (1962) and Hart *et al.* (1963) obtained evidence (presented by Hutchinson (1965)) that seasonal shedding of hair and wool from the legs (from the proximal metacarpus and metatarsus to the hooves) could be attributed solely to changes in daylength. Jefferies (1964) presented similar observations, showing a seasonal reduction in the level of face cover in Merino and Corriedale ewes in Tasmania. Using these examples of moulting of the extremities, Hutchinson (1965), put forward the hypothesis that the shedding mechanism still remains in this part of the fleece, because genetic selection has only acted on the major wool growing portions of the skin. It is considered possible by the present author, that the follicles of these major wool growing portions of skin might have escaped from the controls which hair follicles and shedding wool follicles are subjected to, and become controlled in a similar manner to the epidermal cells from which the germinative tissue was derived embryonically. This is pursued in more detail in the following text.

1.1.6.5. Summary of seasonality.

In summary, it is clear that at least some of the follicles of the modern sheep breeds still possess the capacity to exhibit shedding, and that fluctuations in the rate of growth of fibres occur under natural conditions. The annual rhythms in biological functions are not the topic of interest of the present work, however they do provide some clues to the control of wool growth. Seasonal fluctuations in wool growth rate provide an analogy to the topic of the first experiments reported in this thesis, namely the examination of a possible circadian rhythm in wool growth rate. Finally, the following few words of Ebling (1965) neatly ties Sections 1.1.6.1 to 1.1.6.4 together: "In wild mammals, the necessity of breeding and moulting seasonally has resulted in an integration of the systems; domestication disengages them .....".

1.2. The effects of hormones on wool growth rate.

The following section reviews the influence of the neuroendocrine and endocrine systems on the rate of wool growth. The literature reviewed in this section forms the basis for the experiments carried out using hormones related to stress, such as the catecholamines and cortisol in later chapters.

1.2.1. The neuroendocrine system.

Relatively little information on the effects of the catecholamines on wool growth of sheep has been published and in a review by Wallace (1979a) there are only three references cited, plus reference to unpublished data. All four of these are revisited here along with other references obtained since this review and evidence from another species (*Capra hircus*, the domestic goat).

Ferguson (1949) demonstrated that in animals which had been unilaterally sympathectomised, the annual rate of wool growth was greater on the sympathectomised side. The difference between the two sides could be attributed to the abolition of seasonal fluctuations, and this implicated the sympathetic nerves in the suppression of wool growth. This led to the conclusion (Ferguson, 1949) that sympathetic activity caused vasoconstriction at low temperatures, and that seasonal changes in the rate of wool growth were caused by a resultant lower blood supply reaching the follicles.

On the other hand, adrenalectomised animals maintained on a constant level of mineralocorticoid and glucocorticoid displayed a marked seasonal periodicty in wool growth (Ferguson *et al.*, 1965). This led to the conclusion that corticosteroids (e.g. cortisol see Section 1.2.2 (d)) circulating in the blood do not produce the seasonal modulation of wool growth in this model (Ferguson *et al.*, 1965). Wallace (1979a) used this evidence to suggest that catecholamines of adrenal medullary origin are unlikely to affect wool growth.

Contrary to the observations of Ferguson *et al.* (1965), Reklewska (1975) demonstrated a positive relationship between blood catecholamine levels and wool growth rates. This was only evident in male lambs however, and in concurrently studied ewe lambs no significant correlation was observed. Earlier work which does support the observations of Reklewska (1975) is that of Andersson *et al.* (1963) who found that repetitive cooling of the "heat loss centre" in goats stimulated the growth of the coat compared with untreated animals kept under the same conditions. In their experiments Andersson *et al.* (1963) found that when thermodes were inserted into the skull in the preoptic/anterior hypothalamic region and repeatedly cooled by perfusion with cold water, the growth of that portion of the coat which they refer to as 'wool' (probably the downy undercoat) was stimulated, even though the animals were subjected to warm environmental conditions. However, breeds of goats other than the Angora exhibit an annual shedding of the coat, (and most notably the finer winter coat), and comparison of this species with non-shedding wool growing sheep breeds may not be valid. For these reasons, the evidence for a stimulatory effect of catecholamines on wool growth should be regarded with some caution.

Wallace (1979a) cites unpublished data that show a reduction of L-[^{35}S]cystine incorporation at the site of single intradermal injections of both adrenaline and noradrenaline when compared to a control site. Apart from the fact that the radioactive label was injected intravenously four days after the intradermal injections and wool fibres harvested by plucking another two days later, little detail or data are provided on this experiment. The conclusion drawn in this case was that the catecholamines were causing vasoconstriction and a reduction in wool growth rate in the manner proposed by Ferguson (1949).

Cunningham *et al.* (1979) have since produced evidence contradictory to Ferguson's hypothesis. These experiments capitalised on the ability of wool fibres to incorporate radioactively-labelled cysteine into the forming fibre as in the experiment of Wallace (1979a). Intradermal injections of noradrenaline produced a reduction in the uptake of [^{35}S]cysteine into wool fibres in a dose-dependent manner. In contrast to the earlier conclusions (Ferguson, 1949; Wallace, 1979a) it was shown that the reduction in incorporation of [^{35}S]cysteine was related to uptake of noradrenaline into extravascular sites. To demonstrate the non-vascular

nature of the effect, pure vasoconstricting agents (e.g. angiotensin and ornipressin) were found to have no effect on the uptake of radioactivity. The observed effects were also shown to be due to α-adrenergic receptors since the effect could be blocked by the α-blocker phentolamine but not by the β-blocker, propranolol.

Using short term *in vitro* incubations of skin (sixty minutes), Foldes and James (1981) examined the uptake of [^{3}H]noradrenaline into sheep skin. This study revealed that the uptake of noradrenaline was an active process, sensitive to both cocaine and 6-hydroxy-dopamine but not glucocorticoids. Using this information the authors showed that the radioactive label was essentially taken up by sympathetic nerves and not extra-neuronal tissues. In fact, very little label was taken up by tissues such as vascular smooth muscle and arrector pili muscles associated with the wool follicles. An interesting feature of this paper was that steroid treatment, either of the animal prior to skin removal, or of the skin during *in vitro* incubation had no influence on the incorporation of radioactive noradrenaline, and is one of very few observations which attempt to determine the interaction of the hormones that have been shown to influence wool growth. These observations did not concur with those of Cunningham *et al.* (1979), and considered along with evidence for the control of arteriovenous anastomoses by noradrenergic innervation (Molyneux and Hales, 1979; Foldes and Hales, 1981), may lend further support to the argument for indirect control of wool growth.

Partial sympathectomy of one foreleg of two sheep, and subsequent injections of [^{35}S]cysteine has been shown to produce an increase in fibre length with respect to a matching site on the opposite foreleg (Foldes and Maxwell, 1981). However, an accompanying reduction in mean fibre diameter on the denervated side resulted in no significant overall change in mean fibre volume.

More recently, Williams and Willis (1987) observed that after successful unilateral sympathectomy, the fleece of Wiltshire Horn rams exhibited an increase in the amount of wool grown over a nine month period on the sympathectomised side, a finding similar to that of Ferguson (1949). Although there was no difference in the level of fleece casting between the sides, a significant increase was observed in the number of brush ends formed on the operated

side of the animal. This lead to the conclusion that the sympathetic nervous system may serve a regulatory function in the timing of shedding in the wool follicle. Comparative differences in the histomorphological position of autoflourescent, catecholamine-containing nerve bundles were shown between the two sides. These were suggested to be the "extravascular sites" proposed by Cunningham *et al.* (1979).

From this short review of the effects of catecholamines, it is clear that these neurohormones have some effect on wool growth rate. However, it is unclear whether these effects are completely inhibitory, or stimulatory under some circumstances. It is also unclear whether the effects are entirely due to extravascular sites, or whether there may be some influence exerted by nerve tissue-vascular tissue interaction. There is obviously a deficiency in our knowledge of the effects of the neuroendocrine system, and some of the references given here are either abstracts (Cunningham *et al.*, 1979), or citations of unpublished data (Wallace, 1979a). Williams and Willis (1987) and Andersson *et al.* (1963) made observations on animals that annually shed their fleece, and possible differences between shedding and non-shedding animals should be kept in mind. It is considered possible by the present author that during the evolution of the current wool growing breeds of sheep, the major fleece growing area of the skin may have become divorced from the controls which operate to regulate the growth of hair in other animals.

1.2.2. The endocrine system.

Of greatest significance in terms of the number of hormones assessed is the work of Ferguson *et al.* (1965) who studied the effects of various pituitary hormones and also their relative trophic hormones. This work is referred to by many subsequent authors and is the first to separate the effects of nutrition from those of hormones. Ferguson *et al.* (1965) is also revisited here in an attempt to lay foundations for an explanation of the current understanding of endocrinological influences on wool growth.

1.2.2(a). Pituitary.

Ferguson *et al.* (1965) showed that after a variable post-operative length of time, hypophysectomy stopped wool growth completely in nine ewes. Girard (1969) observed that the cessation of fibre growth after hypophysectomy of Ile-de-France ewes, was accompanied by a change in the histological appearance of the wool follicles. The follicles assumed an appearance similar to that of the resting or telogen stage of growth in animals which exhibit seasonal shedding or cyclic changes in fibre growth rates, while other components of the skin appeared normal, at least under histological examination.

1.2.2(b). Growth Hormone.

Seemingly stimulated by its anabolic effects on muscle tissue, growth hormone from the anterior pituitary, has been investigated with a great deal of interest with respect to wool growth. As with most of the other hormones in this review, some of the earliest work was conducted by Ferguson (1951, 1954) who found that wool growth rate in normal sheep was suppressed by systemic administration of either bovine growth hormone or an extract of bovine pituitaries. Surprisingly, after the treatment period ceased, the rate of wool growth recovered and then increased above pre-treatment levels for an extended period of time. In contrast, when bovine growth hormone was injected into a hypophysectomised ewe, no response was observed, whereas the crude extract was able to produce a response in this animal (Ferguson, 1951, 1954).

The bovine source of the growth hormone, the fact that a crude extract of pituitaries was used and further work (Ferguson and Boyden, 1953) suggested that the effect may have been related to subsequent immunological protection of the endogenous hormone by antibodies evolved toward the exogenous extract. The survival of the hormone in the blood was presumed to have been extended, due to the binding of endogenous antibody during the post-treatment period.

The experiment was subsequently repeated (Ferguson *et al.*, 1965) using both bovine and ovine pituitary extracts. In this work the same effect was observed, suppression of wool growth

during treatment and subsequent recovery followed by stimulated production when either bovine or ovine extracts were used. The suppression of wool production was evident whether or not the hypophysectomised animal was treated with thyroxine, (in this case ovine growth hormone was used). However, no protracted stimulation was observed in the post-treatment period in sheep treated with growth hormone after hypophysectomy. At this time, involvement of an immune response was still favoured by the authors, along with the suggestion of an action or interaction of an "unidentified pituitary factor".

Downes and Wallace (1965) were unable to show any effect of intradermal injections of growth hormone on wool growth either during or after treatment, and this led Wallace (1979a) to suggest that the hormone operates systemically. Wallace (1979a) presented further evidence that growth hormone depresses wool growth during systemic administration, and causes subsequent stimulation for prolonged periods after treatment ceases, although few details of the experiment were provided.

Using a more highly purified ovine growth hormone, Wheatley *et al.* (1966) once again showed the same treatment/inhibition, post-treatment/stimulation response in normal sheep. Extensive metabolic studies were carried out in this work, and based on these additional observations, they suggested that the post-treatment response was due to remobilisation of amino acids preferentially stored in muscle during the treatment period. Thus the immunological effect was rejected in favour of control over the end use of substrate molecules.

Using growing lambs, Reklewska (1974) reported similar findings to other authors, showing increases in wool production at seven and eleven months of age in animals treated with bovine growth hormone every 10 or 15 days until they were weaned at 100 days. It is interesting to note that there was no increase in live weight gain of the treated animals above controls at the completion of the experiment, despite the fact that the treatment significantly increased feed intake.

The interaction of growth hormone and nutritional status has been examined by Wynn *et al.* (1988), who showed a depression of wool growth under an elevated feeding regime but not

under conditions of maintenance feeding. Additionally, these workers studied concurrent changes in a variety of other hormones and substrate molecules, in an attempt to correlate these with wool growth rate depression. They showed that plasma growth hormone was substantially increased by daily injection of the purified hormone. This appears to be due to an increase in endogenous levels and residual exogenous hormone (Wynn *personal communication.*). Growth hormone treatment also resulted in increases in the circulating levels of insulin, somatomedin C, free fatty acids and glucose, without significant changes in thyroid hormones, cortisol, prolactin or amino acids.

Although Wynn *et al.* (1988) have been able to repeat the results of the previous authors, it should be noted that the experiment was carried out under conditions of continuous light and in work reviewed in Chapter Two, the concentrations of a number of hormones have been shown to vary throughout the day in animals kept under a variety of diurnal lighting regimen. It is also important to note that some workers have reported rapid falls in growth hormone levels after feeding (Bassett, 1974a and b) and others have observed similar rapid changes in animals anticipating feeding (Tindal *et al.*, 1985). In the studies of Wynn *et al.* (1988), growth hormone was determined in plasma samples collected half an hour before the daily injection (i.e. 23.5 hours after the previous injection). It is unclear which case would hold in these animals, but it is possible that the plasma samples were taken during a period of rapid changes in the levels of growth hormone which may have influenced the results.

To conclude, it seems that growth hormone has a depressant effect on the rate of wool production and this review has been included primarily to highlight the interaction of growth hormone with the catecholamines. It seems that this hormone may cause increases in the rate of muscle deposition, at the expense of wool production. When animals are fed (Bassett, 1974a and b), or are expecting a feed (Tindal *et al.*, 1985), plasma growth hormone levels decline, and a fall in growth hormone levels can also be produced by the administration of catecholamines (Hertelendy *et al.*, 1969; Bassett, 1971). More work on the levels of growth hormone and this interaction are reviewed in Chapter Two and this section is designed primarily to introduce the effect of the hormone.

1.2.2(c). Thyroid Hormone.

Ferguson *et al.* (1965) showed that supplementing hypophysectomised sheep with thyroxine or thyroid stimulating hormone on a daily basis restored wool growth to pre-operative levels. However, there was no increase in the rate of wool growth above the original level of production when large doses of either thyroid stimulating hormone or thyroxine were injected into animals that had been hypophysectomised.

Treatment with thyroxine was able to completely restore wool growth rates in thyroidectomised animals as it does in hypophysectomised animals (Ferguson *et al.*, 1965). A 40% reduction in wool growth rate was demonstrated in sheep that were thyroidectomised but received no replacement therapy. The normal rate of wool growth was maintained if only a small fragment of thyroid tissue remained. In contrast to hypophysectomised animals, these animals exhibited slight increases in output of wool when doses of thyroxine above that which restored normal levels were administered.

Hart (1954) and Godfrey and Tribe (1959) had previously shown that thyroxine was important in wool growth, and in these early experiments demonstrated that in sheep that were fed at a controlled level in pens, wool production could be stimulated by implants containing the hormone. Following on from this work, Coop and Clark (1958) demonstrated that a significant effect occurred only when implantation of the hormone coincided with the seasonal winter depression in wool growth. This observation, coupled with the work of Henneman *et al.* (1955) who showed a seasonal fluctuation in thyroid secretion rates, led to the hypothesis that thyroxine was responsible for the seasonal variations in wool growth rate. This was subsequently refuted by Wallace (1979b) and Ryder (1979) who showed that the seasonal fluctuations in wool growth and in thyroxine concentration were poorly correlated.

More recently Maddocks *et al.* (1985) have shown that wool growth in thyroidectomised animals can be fully restored when plasma levels of thyroid hormones are maintained at 30% of normal levels. In this experiment, only minor increases in wool growth occurred with increases in circulating thyroxine levels up to 300%. From this evidence Maddocks *et al.*

(1985) claimed that seasonal fluctuations in the rate of thyroxine secretion would not produce a seasonal change in the rate of wool growth, since throughout the seasons, levels of thyroxine observed by Ryder (1979) and Wallace (1979b) never exceeded these limits. When using fibre length and diameter measurements, Hynd (1989b) found that circulating plasma thyroid hormone could be depressed further to 15% of "normal" without influencing wool growth.

Local intradermal injections of thyroxine have produced local increases in length growth rate of the fibres over the site of injection, in normal sheep (Downes and Wallace,1965). This response was not uniform, and some fibres were stimulated whilst others remained unaffected, and yet in other fibres a break or weak point in the fibre was produced.

As a result of these observations, thyroid hormones are now believed to have a facilitatory role, rather than a regulatory role in wool production (Hynd,1989b). That is when thyroxine is not present, wool growth will continue at a much reduced rate, but there is no correlated or dose dependent increment in fibre production with circulating concentrations ranging from below normal to above original levels.

1.2.2(d). Corticosteroids.

Adrenocorticotrophic hormone has been shown to depress wool growth rate in sheep maintained on a constant level of food intake (Lindner and Ferguson, 1956). Ferguson *et al.* (1965) observed that when wool growth recovered from depression caused by adrenocorticotrophic hormone, a weak point or "break" in the wool fibres became evident, and some fibres were completely shed. After severe cases of stress imposed either naturally, experimentally or by management changes on many breeds, the growth of fibre is also known to slow or even cease and the fibres to shed (Donald, 1979). Lindner and Ferguson (1956) suggested that the mediator in many of these examples may be changes in plasma concentrations of hydrocortisone (or cortisol). The similarity of the "break" caused by adrenocorticotrophic hormone to "tenderness" in the wool of sheep subjected to stress in the field led to the suggestion that adrenocortical activity was the mediator of this response.

Castor and Baker (1950) showed that prolonged topical application of adrenocortical hormones produced histological changes in the skin of rats, including cessation of hair growth, a reduction in epidermal thickness and sebaceous gland size. Modifications to the dermis were also evident, including reductions in dermal thickness, amounts of collagen and elastin and in the number of fibroblasts present. A reduction in the output of wool compared to a control site was observed by Lindner and Ferguson (1956) after applying 9-α-fluoro-cortisol to the skin of three sheep, although a smaller response was observed when cortisol was used. In its response to corticosteroid hormones, wool-growing skin therefore behaves similarly to hair growing skin.

Cortisol was found to be the major corticosteroid present in sheep plasma (Bassett and Hinks 1969) and circulating levels of 1 to 10 ng/ml were determined in unstressed sheep. In animals unaccustomed to handling it was also found that the level of this steroid was elevated within ten to twenty seconds by the act of sampling, and increased further over the following fifteen minutes. Chapman and Bassett (1970) showed that plasma levels of up to 30 ng/ml were sufficient to reduce wool growth in animals on a restricted intake of feed. When feed intake was not limited, a moderate increase in plasma cortisol to around 10 to 12 ng/ml stimulated the growth of wool. In addition, the retrogression of other skin components was similar to that observed in rats (Castor and Baker, 1950) and sheep (Lindner and Ferguson, 1956). Chapman and Bassett (1970) went on further to say that cessation of fibre growth was accompanied by the formation of "brush-ends", a trait normally associated with cessation of hair growth (Auber, 1950) and wool growth in the shedding breeds of sheep (Ryder and Stephenson, 1968). This would appear to be further evidence suggesting the regulation of wool growth by corticosteroids is similar to that of hair growth.

Intradermal doses of cortisol have reduced the length growth rate of individual wool fibres (Downes and Wallace, 1965). However, this response was not reflected by all wool fibres in the same sample; some showed a complete cessation of growth while others showed no significant effect. Indeed at low intradermal doses of cortisol (3μg), a stimulation of the growth of wool was observed. As with intradermal injections of thyroxine, this was not true of all fibres in the one sample and the effects ranged from stimulatory to inhibitory.

In his review, Wallace (1979a) provided unpublished results of A.L.C. Wallace and A.M. Downes which showed that intradermal injection of cortisol reduced the incorporation of [^{35}S]cystine into wool fibres. The radioactive label was administered intravenously, on day seven, after one week of intradermal doses (12.5 to 1600 μg) of cortisol (injected on days 1,4,5,6,7). Wallace (1979a) also stated that unpublished results of K.A. Ward suggest that cortisol has no effect on wool follicles under conditions imposed on *in vitro* cultures of wool roots in the short term (a few hours). Ward and Kasmarik (1979) in work published since this review have provided evidence of these studies, showing that cortisol is metabolised to cortisone by sheep skin in culture, however they make no comment on whether any effects were observed on the rate of keratin synthesis *in vitro* (an indicator of wool growth rate) in their studies.

An interesting recent development is the study of the effects of epidermal growth factor on wool growth rate. In the context of the present work, it is important to note the observations of Singh-Asa and Waters (1983) who showed that intravenous infusion of defleecing doses of epidermal growth factor caused a seven fold increase in cortisol levels to 70 ng/ml, which in the light of the work by Chapman and Bassett (1970) could be adequate to produce defleecing on its own. However, since the experiments were primarily aimed at defleecing sheep with the compound, culminating in the recent paper by Panaretto *et al.* (1989), little work has been directed at the effects of physiological concentrations of epidermal growth factor on the growth of wool. There is also contradictory evidence on the level at which the compound exerts its effect. The earliest works involved the use of intravenous injections of defleecing doses of the growth factor (Moore *et al.*, 1981,1982; Panaretto *et al.*, 1982). However, McDonald *et al.* (1983) were unable to produce a local effect when infusing the hormone into an artery which serves a defined area of skin. On the other hand, Chapman and Hardy (1988) were able to demonstrate a defleecing type of response in skin treated by intradermal injection with epidermal growth factor. Thus it is not clear whether the action of this growth factor occurs at the local level or the whole body due to interactions with other hormones.

From this body of work, it would appear that the glucocorticoid hormones have quite profound effects, not only on wool growth but also on the entire skin. The dose-response-feed intake

interaction has been examined (Chapman and Bassett, 1970) but little work has been directed at the minimum time taken to elicit a response. In a comment to this effect, Wallace (1979a) states that **"No clear information is available on the time that different plasma concentrations of cortisol must be maintained to stop wool growth."**

1.2.2(e). Prolactin.

Wallace (1979a), using evidence from hypophysectomised (Ferguson *et al.*, 1965) and normal animals (Wallace and Wheatley, cited as unpublished by Wallace (1979a)), concluded that prolactin has no effect on wool growth rates. Wallace (1979a) also noted that prolactin levels in sheep vary throughout the year in a manner which appears to be related to the ambient temperature (Radford, cited as unpublished in a review by Wallace (1979a)). Ravault (1976) concluded that daylength had a major influence on prolactin levels, but also considered that temperature had a minor influence, which could not be ignored.

In contrast to the conclusions of Wallace (1979a), Downes and Wallace (1965) had previously shown a depressant effect of prolactin when administered intradermally to one sheep. It was shown that at a low dose rate (5 μg) and a very high dose rate (625 μg) wool growth was stimulated, yet it was inhibited by intermediate doses (25 and 125 μg). However in these early experiments the effects had proven to be variable both between dose rates, and between fibres within the one dose rate treatment. This seems to have been the case with many of the other hormones studied (see Section 1.2.2 for relevant details) and even intradermal injections of saline alone produced an increase in the rate of wool growth (Downes and Wallace, 1965). This may have given the latter author reason to doubt his own earlier evidence, or it may have been disregarded due to the small numbers of sheep studied. (If this is the case, then Wallace (1979a) has not been consistent in this regard, as evidence with respect to growth hormone from Downes and Wallace (1965) was used in the same review. Experiments carried out in this laboratory (Hynd and Everett, unpublished) have provided strong evidence to suggest that intradermal injections of saline have no effect on fibre growth rate)

Despite the dismissal of this hormone by Wallace (1979a), prolactin has been included in this review since stress is known to elevate the levels of prolactin in dairy cows (Raud *et al.*, 1971). Lincoln *et al.* (1980) concluded that the effects of prolactin and growth hormone on wool growth remained to be elucidated. Furthermore, Lincoln (1990) has shown that seasonal rhythms in prolactin levels are correlated with the resurgence of growth of the coat in the more primitive breeds such as the Soay and Wiltshire, but not in wool-growing breeds such as the Merino.

1.2.2(f). Melatonin.

Melatonin is intimately related to seasonal cycles in reproduction by signalling changes in daylength (Kennaway and Gilmore, 1985; Arendt, 1986), however the effect of melatonin on the growth of the fleece appears to depend on whether the breed studied is a 'primitive' or 'modern' wool-growing breed. Williams (1981) found that removal of the source of melatonin, the pineal gland, altered the timing of shedding cycles of Wiltshire Horn ewes. Similarly, pinealectomy prevented cessation of fibre growth from primary wool follicles of Limousine rams (Rouget *et al.*, 1984). Lincoln *et al.* (1980) observed that bilateral superior cervical ganglionectomy of Soay rams, (which effectively pinealectomises the animal), abolished changes in the rate of growth of the pelage, evident in control rams which experienced similar lighting conditions.

Implantation of constant release implants into Soay rams has been shown to induce moulting during periods of long days (Lincoln and Ebling, 1985). In contrast, no significant difference in wool growth rate was observed in either the Romney (Harris *et al.*, 1989) or the Merino (Foldes *et al.*, 1990), when treated with melatonin implants during periods of long days. It is therefore apparent that melatonin, like prolactin (Section 1.2.2(e) above), is important in the wool growth of shedding or 'primitive' breeds, but not wool-growing breeds.

1.2.2(g). Other hormones under pituitary control.

A number of hormones have been lumped into this section, primarily due to the lack of effects on wool growth observed when physiological levels of the hormones are considered. The best example to illustrate this is the sex hormones; in a review by Wallace (1979a) the author summarised the effects of the gonadotrophins and the sex hormones using the work of Ferguson *et al.* (1965) to show that at physiological levels, the sex hormones have little effect on the rate of wool growth. Slen and Connell (1958) had demonstrated that large doses of testosterone stimulate and oestrogen inhibit wool growth.

1.2.3. Summary of the effects of the various hormones.

Many of the hormones outlined above have the potential to influence the rate of wool growth under a variety of conditions. Both the endocrine and neuroendocrine systems have been observed to affect wool production. The experimental techniques used to measure these effects are examined in the following section. Chapter Two returns to the topic of hormones, because there are methods of measuring rapid changes in hormone levels, and until recently an equal sensitivity in measurement of the rate of wool growth.

1.3. Accuracy and limitations of various techniques used to measure wool growth rate.

The review by Wallace (1979a) is often quoted in this literature review, but the techniques available at that time have since been improved upon, and it is thus time that the work be re-examined to determine, among other parameters, the minimum time necessary for a response to be elicited by the hormones previously tested. As will be shown in the following section, advances in the methods of measuring wool growth rates have now begun to catch up with the methods of detecting changes in the levels of plasma hormones. In 1985, Maddocks *et al.* (1985) were still using the age-old tattooed patch and dyebanding techniques, alongside modern radioimmunoassay techniques for the detection of small changes in hormone levels. The main aim of the present study is to update the literature with respect to the effects of hormones, and primarily as the title suggests, the stress hormones.

In all of the examples outlined in Sections 1.1 and 1.2 there remains one underlying deficiency. The deficiency lies in the methods used to index rates of growth of the fleece, or comparative changes in this growth rate, which are limited in terms of the time taken to measure a response to a given stimulus. The basic methods, and the deficiencies of each are considered below.

1.3.1. Antique techniques.

The slowest method is the "tattooed patch method", which involves harvesting the wool grown over a certain period of time, from an area of skin delineated by tattoo lines (Langlands and Wheeler, 1968). It is necessary to leave the patches for at least ten days between successive harvests, and thus any treatment must be imposed for a relatively long period. Unfortunately there is a delay between the production of a given portion of the fibre and that same portion protruding from the skin to a level where it can be harvested. This delay is of course dependent on the rate of fibre production, and as a result the relative differences become buffered and reduced and the accuracy of the method is thus limited. The worst possible case of this is where fibre growth has ceased (e.g. Lindner and Ferguson (1956)); once the wool begins to grow again there is a lag between resumption of growth and the collection of wool by

harvesting patches. Despite these errors, this method is one of the simplest and as a result, it has been, and still is, used extensively in wool growth experiments.

Perhaps the next most popular techniques are those of dyebanding (Langlands and Wheeler, 1968) and autoradiography of fibres (Downes *et al.*, 1964). The first of these is as the name suggests, simple marking of the fleece at the skin surface using a dye or sometimes paint, and measuring the distance between successive bands spaced at a given length of time. Autoradiography of wool fibres using radioactive sulphur compounds is essentially the same as dyebanding except that it involves labelling the wool fibre closer to the base of the follicle, in the keratogenous zone. [^{35}S]cysteine is injected into the animal and becomes incorporated into the keratin proteins of the wool, which can then be harvested and autoradiographed and the distance between two consecutive injections measured by microscopy. Initially, whole body labelling was undertaken using large intravenous doses of radioactive compounds, (Downes and Lyne 1959; Rougeot,1959). More recently small intradermal injections have successfully been used to label the fibres over the site of injection (Downes *et al.* 1964). Dye banding is subject to errors similar to those of the tattooed patch method, i.e. buffering of the measured response.

Some workers have demonstrated that fibre length may change without a concurrent change in diameter or vice versa (James, 1963; Reis and Tunks, 1978; Downes and Wallace, 1965; Woods and Orwin, 1988). Diameter must therefore be measured concurrently, on segments of the wool which are delineated by the dyebands or radioactive markers, to determine the volume of wool produced over a given period. If not, changes in total wool production may not be truly reflected in experiments which measure length changes alone. The same consideration is important if total changes in the length and diameter occur over different lengths of time, as would appear to be the case with growth hormone treatment (Wallace, 1979a) or abomasal infusion of zein protein (Reis and Colebrook, 1972).

1.3.2. Short term techniques.

The shortest measurement period of fibre growth possible with the methods outlined in the previous section is about three to five days (depending on the rate of growth) using intradermal [^{35}S]cysteine and autoradiography. To more precisely identify the time sequence of responses, a number of methods for the detection of progressively shorter term changes in wool growth rates have been developed. These involve both *in vivo* and *in vitro* techniques and are reviewed here with special reference to the minimum time to measure a response to a treatment permitted in each case. It is important to note that despite the fact that these techniques allow a shorter time frame and in some cases are easy to use, they often measure effects in only one compartment of wool growth and therefore the relevance of the results to actual wool production varies.

1.3.2(a). *In vitro* short term methods.

Skin strip and bulb culture techniques.

Techniques have been developed for use in the study of the response of wool follicles to various *in vitro* treatment regimes (Ward and Kasmarik, 1979; Ward and Harris, 1976; Ward, 1976). There are two basic methods, the first, a 'plucking' method, was originally developed by Wilkinson (1970) and the second, a short term *in vitro* culture of whole skin (Ward and Harris, 1976) or wool root cells only (Ward, 1976). After removal from the animal, the tissue is placed in culture media containing varying levels of hormonal, defleecing or nutritive compounds, for different time periods (0 - 22 hours). A radioactive labelling compound is also included in the medium, and it is the incorporation of this label which is compared between treatments. Any of these methods can be used to measure DNA, RNA or protein production by adding radioactive precursors of the nucleic acids (thymidine or uridine) or amino acids to the culture medium.

Downes and Wilson (1971) using a slightly different plucking technique showed that radioactive [^{14}C]leucine was effectively incorporated into plucked follicles for incubation times

of up to twelve hours. These workers provided autoradiographs which showed very effective accumulation of the leucine into the follicle bulb and claimed that very little was incorporated into the inner or outer root sheath material that remained with the bulbs after plucking. Plucking techniques unfortunately remove most of the dermal tissue and thus any interaction between these tissues and the bulb tissue is lost. On the positive side, it is feasible to to measure differentials in incorporation of radioactivity down to the level of sensitivity of the isotope detection method, and small doses may be used inexpensively and quickly to screen many samples.

The 'skin strip' method of Ward and Harris (1976) involves the use of whole skin in thin strips, and using autoradiography these authors showed that 88% of the radioactivity incorporated into the skin was found in the wool follicle and very little in the surrounding dermal and epidermal tissues. This lends support to the observations of Downes and Wilson (1971) and thus makes this method just as suitable as plucking for the study of wool follicle responses to different compounds. If there is reason to believe that there may be some interaction between the surrounding tissues, the follicle bulbs and the treatment imposed, then this would be the preferred method. Dermal/epidermal/follicle contacts remain intact, and approximately equivalent proportions of these tissues are present in the culture as *in situ*. Once again, relatively short term comparisons are possible, however both of these methods are divorced from changes in the rest of the animal. If a hormone is removed rapidly from the skin, or never leaves the blood stream within the skin then the following *in vivo* techniques would be the methods of choice. The major limitation to consider when using the *in vitro* techniques is the length of time required to deplete endogenous substrates and the lag between substrate application and the follicle response.

1.3.2(b). *In vivo* methods.

Incorporation of radioactivity.

A modification of the autoradiography technique was pioneered by Downes and Wallace (1965) and used by these authors (Wallace and Downes (1979) cited as unpublished by Wallace (1979a)). In this case the level of uptake of L-[^{35}S]cystine into fibres plucked from the skin was measured. For example after four days of intradermal administration of hormones, (catecholamines mentioned in Section 1.2.1. above), L-[^{35}S]cystine was injected intravenously and fibres plucked from the skin (also cited as unpublished by Wallace (1979a)). Changes in wool growth rate caused by the hormones were determined by comparison between the amount of radioactivity incorporated at treated and control sites. The minimum treatment period in this case was four days, and this seems to have been adhered to in the experiments of Cunningham *et al.* (1979) who have since used the technique.

The colchicine technique.

Schinckel (1961, 1962) introduced a method of measuring wool follicle responses *in vivo* over comparatively short periods of time. The technique involved the intravenous administration of a bolus dose (0.15 mg/kg body weight) of the alkaloid colchicine (Schinckel, 1961, 1962; Short *et al.*, 1965; Wilson and Short, 1979). This compound has the power to bind reversibly to tubulin polymers and thus arrest the formation of spindles during cell division (Borisy and Taylor, 1967a and b). Arresting spindle formation effectively prevents cell division from proceeding beyond metaphase and has been applied to a number of situations including the destruction of tumors, the production of polyploids in plant breeding and also to determine mitotic rate in cell culture systems.

The colchicine technique can be summarised as follows: Several hours after injection of the colchicine, samples of skin were taken, processed and selectively stained to highlight arrested mitotic figures, according to the method of Clarke and Maddocks (1963). After such treatment it is possible to count the number of arrested mitoses in each follicle, and Schinckel (1962) has

shown **the number of cells arrested per follicle bulb to be directly proportional to the rate of wool growth**. With this method it is possible to determine rates of wool growth over much shorter time periods than with the radioactive *in vivo* methods, i.e. over a few hours. The drawback with this method is that the alkaloid will stop cell division non-selectively, and as a result other vital processes of the body are interrupted. Cell division processes such as those in the bone marrow are also inhibited and the general toxic effect of the compound means that survival of the animal is often compromised. Thus, under sequential sampling regimes, this method is less useful than patch harvests or autoradiography, because the effective dose for complete arrest is very close to the lethal dose. For example, seven out of ten sheep died when high doses of colchicine were injected during the experiments of Wilson and Short (1979).

On the basis of these experiments and using the same logic as Downes *et al.* (1964) (see Section 1.3.1. above), Hynd *et al.* (1986) have modified the method of Schinckel (1961, 1962) to an intradermal technique. A fine gauge needle was pushed gently into the dermis and a very small dose (10 to 100 μg) of colchicine injected into this tissue in a low volume of carrier (0.1 ml saline). A sample was taken directly over the injection site 2-4 hours later and processed as with the method above. Hynd *et al.* (1986) have shown that the **cell division rate thus measured is correlated with the rate of wool growth provided the dose of colchicine is sufficient**. This technique thus provides us with an accurate method of measuring follicle responses over periods of two to four hours, which could be followed by a second period of sampling after some other treatment. For example, this technique has been used by Hynd (1989b) to demonstrate a reduction in the rate of cell division accompanying the drop in plasma levels of thyroxine, post-thyroidectomy (Section 1.2.2(c)). A possible flaw in the technique is that the base line or control rate of cell division may not remain constant over a given period. There is a body of literature which shows that this is indeed the case in other tissues and some of this work is reviewed in the following chapter, along with similar evidence for follicle bulb cell division. Another problem is the toxicity of the compound to the cells themselves, quite apart from the toxicity to the whole animal, and some facets of this are explored in Appendix One.

The definition of Cell Division Rate.

Throughout this thesis, **Cell Division Rate** (abbreviated to **CDR** in all figures) has been generally defined as: The average number of cells per random follicle section, of a given population of follicles, arrested by colchicine at metaphase of mitosis during a given period of time (usually two hours). The cells arrested at metaphase are those darkly stained with haematoxylin and eosin stain in a 10 μm thick histological section through the longitudinal axis of the follicle bulb. The average was determined from a frequency distribution of the number of darkly stained cells counted per follicle bulb. (excluding those visibly contained in the outer root sheath). Plate 1.1 portrays one such follicle and the frequency distribution determined for the population from which this particular follicle was drawn is shown below it (Figure 1.3). Clearly the number thus obtained is the average number of cells arrested during division, and not the rate at which the cells complete the division cycle. Indeed, recent evidence from this laboratory (Hynd and Everett, 1990) suggests that colchicine, being a mitotic inhibitor, produces a lower estimate of the rate of cell division than other techniques.

The following reasoning provides the justification for the continued use of this method throughout the course of the experiments conducted for the preparation of this thesis. Analysis of all experiments contained in the present studies were carried out by comparing treatment cell division rates with control cell division rate. Both Schinckel (1961, 1962) and Hynd *et al.* (1986) have shown that cell division rates determined using colchicine are well correlated with wool growth rate. It is therefore assumed with confidence, that the arrest of cells using the colchicine technique provides a good qualitative estimate of wool growth rate, suitable for statistical comparisons. The effects of the mitotic inhibitor should be relatively the same for each sampling site on an individual animal, provided the skin is similar and the drug is administered in a consistent manner. Finally, since inhibition of mitosis would tend to yield estimates lower than actual cell division rates, there would be a tendency to underestimate the magnitude of the difference between two populations of follicles.

1.3.3. Summary.

It would be possible to harness all of these methods at once and screen one hormone for its effect on different systems or components of wool growth as follows:

follicle activity *in vitro*	Bulb
skin culture *in vitro*	Bulb-dermal tissue interaction
cell division rate *in vivo*	Bulb-dermis-skin and blood interaction
radio-label incorporation *in vitro*	Keratin formation
radio-label incorporation*in vivo*	Skin and keratinisation interaction
fibre produced	Whole body effects including metabolism, excretion, blood flow etc.

The cell division and keratinisation components of wool growth are separable using these screening procedures. Thus it is now possible to detect at which level or compartment of fibre production a given hormone will influence wool production. Some of these techniques were employed in this thesis to determine where the stress hormones exert an influence on wool growth.

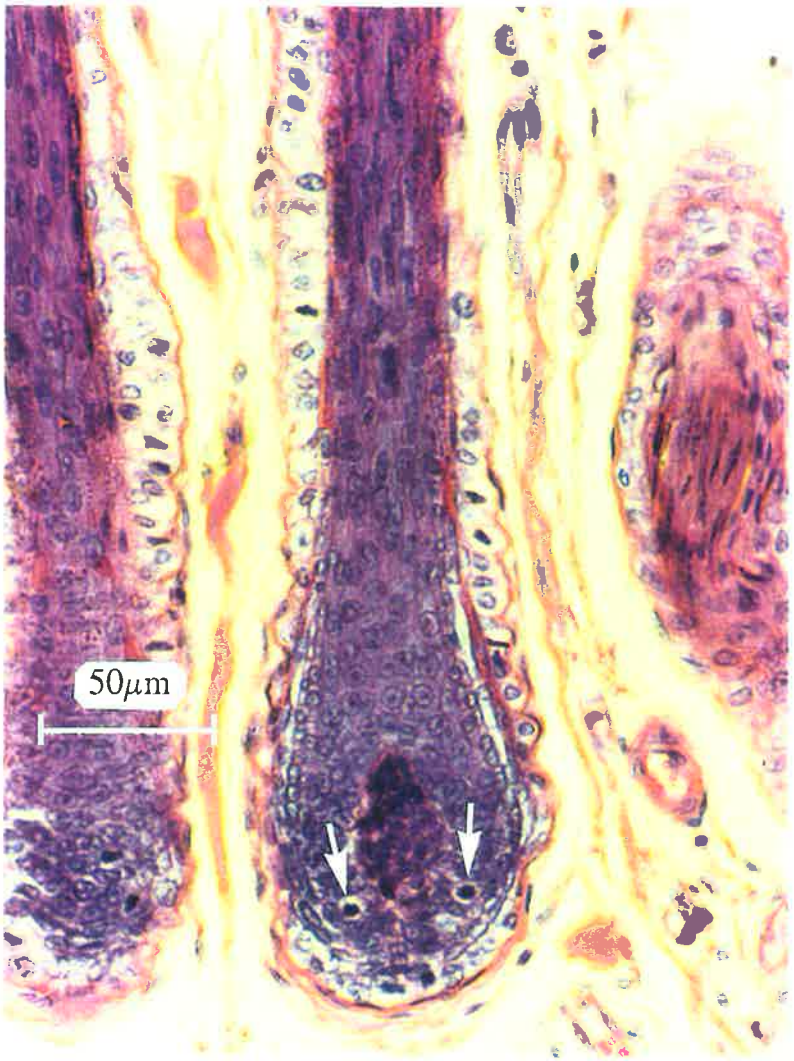


Plate 1.1. A photomicrograph of a histological section through a wool follicle bulb. The cells arrested at metaphase by colchicine are indicated by the arrows.

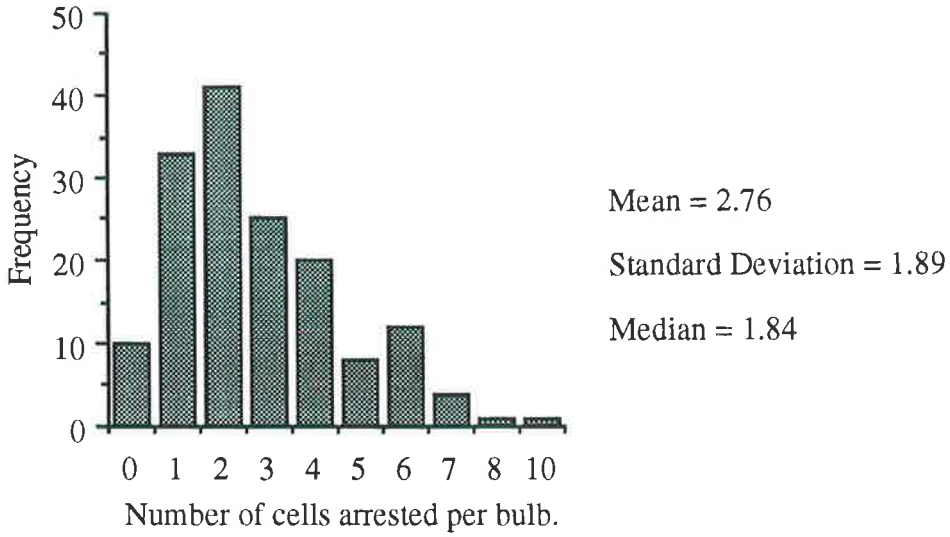


Figure 1.3. The frequency distribution of the number of cells arrested per average random bulb section, and the mean, standard deviation and median calculated from this distribution. Plate 1.1 depicts one such random follicle.

1.4. Conclusions and implications of this literature review.

From the prior discussion, it can be seen that wool growth is a modified pelage growth process. At least on the main wool-growing areas of modern sheep breeds, it would appear that the growth of the fleece is a continuous process (i.e. in a constant state of anagen). Annual casting of the fleece is a trait which has essentially been lost through selection for increased wool production. However, shedding of the fleece still seems to occur even in the "most advanced" wool-growing breeds, and Merinos left in a feral state for over one hundred years appear to have regained the ability to shed their fleece (Orwin and Whitaker, 1984). Whole body fleece casting can be "awakened" in the non-shedding breeds when treated with defleecing levels of corticosteroids and their analogues, as evidenced by the formation of brush ends in the fleece during the experiments of Chapman and Bassett (1970). The same would appear to be the case with a range of defleecing chemicals available, although others do not produce hair-like shedding events. It is interesting to note that the extremities may remain the difficult areas to remove the wool from as shown in the record of one of the earliest incidences of chemical defleecing (Hughes, 1959). Panaretto *et al.* (1975) made similar observations when using cortisol analogues and this evidence coupled with the comments of Hutchinson (1965) (Section 1.1.6.4. above) imply that the wool growing areas of the skin may have escaped from normal hair growth controls and become subject to controls similar to the epidermis. (Note: The literature on the chemical defleecing of animals has essentially been ignored in the present work, since most of the chemicals are foreign to the body of the sheep. The animal could thus be regarded as being in a diseased state, and this thesis is more interested in the stress physiology of wool growth.)

Although wool-growing breeds of sheep have become non-shedding, many of them remain seasonal with respect to reproductive characteristics and most show an annual rhythm of wool growth rate without exhibiting shedding. The control of this rhythm has long been the subject of research and debate, primarily because the loss of potential production could comprise a considerable loss to an industry worth over 6 billion dollars to Australia alone (ABAE, 1988). Factors such as nutrition, thyroid hormones, cortisol and catecholamines have been systematically suggested and disproved as the responsible mediating agent in this annual

rhythm. Finally, in the shedding breeds it appears that responsibility for the control of this rhythm has come to rest on the response to a daylength stimulus, which entrains an inherent rhythm that becomes free running after removal of the stimulus. It is still unclear whether this is the case in non-shedding breeds and further research is needed.

With this information in mind, and the large body of research to be updated, the questions approached in the latter part of this thesis are along the theme of: "Does a given stress hormone produce a change in the rate of cell division in wool follicles, within a certain time period?" However, to determine the short term effect of any stimulus on cell division, it was deemed necessary first to elucidate and then index whether there are any naturally-occurring changes in the normal level of this parameter over the same period. For example if we wished to detect the effect of thyroxine two or three hours post injection, then it was obvious that there must be no change in the basal level of cell division over this same period. There are two ways to overcome this problem; the first is to use control versus treated sites on the one animal, the second is to determine whether the fluctuations exist and then to design the experiment and interpret the results with this in mind. Preliminary evidence and parallels in other tissues suggest that there may in fact be some fluctuation in cell division rate throughout the day, and these are reviewed in Chapter Two, which leads into the first experiment.

Chapter Two.

Fluctuations in the rate of cell division and related processes in various tissues.

2.1. Introduction.

From Chapter One, it is clear that the rate of pelage growth is influenced by a number of regular physiological events and physiological perturbations that occur in response to external stimuli. The first part of the present chapter is similarly devoted to a review of literature, however the topic is much more concise. The present chapter deals with circadian rhythms, and especially those related to cell division rates and hormone levels. A body of evidence which shows that mitotic rate varies with respect to the time of day in a number of tissues has been summarised in the following text. Hormonal fluctuations with respect to some timing cue; either time of day, period of activity or time of feeding are also reviewed. A small amount of literature which suggests that wool follicles exhibit a circadian fluctuation in mitotic rate, is also presented.

In reports of initial work on biological rhythms in wool follicle mitotic activity, the rhythms were referred to as "diurnal" (Hynd *et al.*, 1986; Scobie and Hynd, 1987); however "circadian rhythm" is now considered to be a more appropriate term. The following definitions are taken from the Macquarie Dictionary and summarise the reason for the change in terminology, since mitotic activity was higher during the night rather than the day.

circadian *adj.* designating physiological activity which occurs approximately every twenty-four hours, or the rhythm of such activity.

diurnal *adj.* 1 of or pertaining to each day; daily. 2 active by day, as of certain birds and insects.

It was considered possible that the changes in mitotic rate could be caused by circadian fluctuations in hormones which have demonstrated the capacity to influence wool growth rate over longer periods of time (reviewed in Chapter One). On the basis of this collection of evidence, an experiment was designed and conducted to confirm the earlier observations and provide some insight into the factors which might create the rhythm. This experiment is reported in the later sections of this chapter.

2.2. Cell proliferation in rodent epithelial tissues.

It is noteworthy that the pelage of rodents exhibits a cyclical hair replacement over the entire body (Chase, 1965). This occurs in waves that proceed over the entire surface of the animal, but do not replace all of the pelage in one pass of a wave (Johnson, 1965). That is, a sequence of separate shedding phases will occur before all of the fibres in a given area are replaced.

As far as hormonal control of cell division rate in rodent tissues is concerned, much work has been undertaken, and reviewed (Bullough, 1965; Ebling, 1965). Ebling (1965) provided examples of systemic factors which control the shedding cycles of rodents, but also presented an interesting diagram which shows the relation of the various glands to the hair growth cycles. This diagram is repeated here as Figure 2.1, and although the author claimed that Merino sheep show virtually no cycles in activity at all (Ebling, 1965), it is interesting to note that if we change the interpretation of the diagram from shedding cycles to rates of growth, the diagram becomes readily applicable to wool growth from the follicles of the sheep. (As shown in Chapter One, the gonads have little significant effect on wool growth, and the magnitude of the arrows would need to be adjusted accordingly. Other hormones reviewed in Chapter One have not been considered in Figure 2.1.)

Circadian fluctuations in the rate of cell division have been demonstrated in the tissues of many organisms and some of these have been reviewed by Bullough, (1962), Sigdestad *et al.* (1969) and Bertalanffy, (1960). A circadian periodicity has not been demonstrated in the rate of hair growth in the rodent, however such a rhythm has been observed in the rate of mitosis of rodent epidermal cells (Bullough, 1965). In general these fluctuations roughly follow a sine curve pattern, with a peak in the middle of the day and a decline in the rate to a low in the middle of the night or early hours of the morning. Sigdestad *et al.* (1969) suggested that the daily rhythm in intestinal cell division may be related to the time of feeding rather than the light-dark cycle. Bullough (1962, 1965) suggested that the sleeping and wakeful activity of the animal may be the possible cause of the fluctuation in mitotic rate

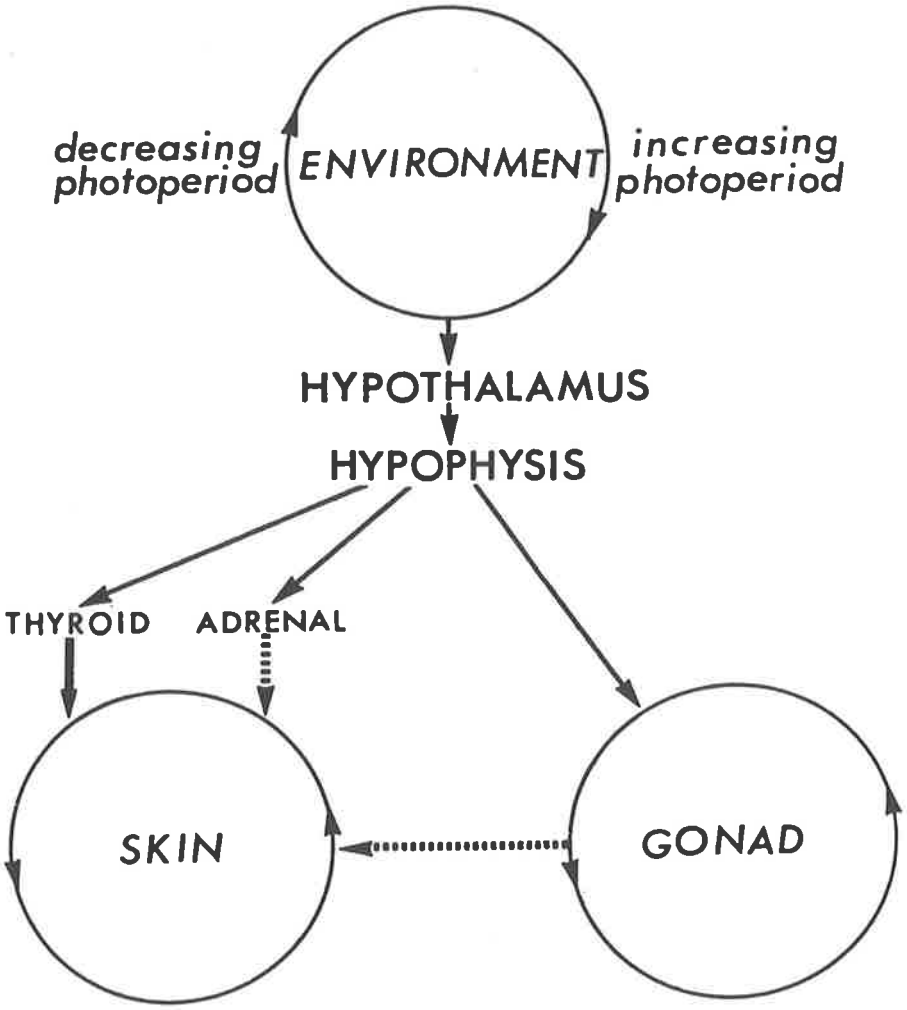


Figure.2.1. Schematic representation of relationship between environmental, gonadal, and skin cycles. Unbroken arrows represent pathways of acceleration, broken lines pathways of inhibition. Taken from Ebling (1965)

Bullough (1965) reviewed work that showed cell populations with either a very high or very low mitotic rate exhibited no evidence of a circadian rhythm of mitosis, whereas a circadian rhythm could be demonstrated in many tissues with an intermediate mitotic rate. In this review, Bullough (1965) concluded that the active or anagen hair follicle belonged to the first group, as it had been found to exhibit no mitotic rhythm.

Using tritiated thymidine labelling and subsequent autoradiography, Pilgrim *et al.* (1963) followed on from the work on cell division rates to show that fluctuations in the rate of mitosis in many rodent tissues were accompanied by fluctuations in the rate of synthesis. The pattern

was similar for cell division, but roughly twelve hours out of phase in the twenty-four hour period. These workers observed that the rate of cell division showed circadian periodicity in the oesophagus, fore-stomach, abdominal skin epidermis and tongue strata basale. However, in the rapidly dividing cells of the crypt epithelium in the jejunum, there was no circadian fluctuation evident.

In contrast, Sigdestad *et al.* (1969) were able to demonstrate a circadian rhythm in synthetic rate of some tissues which Pilgrim *et al.* (1963) had previously claimed did not possess them. It was suggested by Sigdestad *et al.* (1969) that the results of Pilgrim *et al.* (1963) could have been an effect of the choice of sampling time, which caused them to miss the peak in DNA synthesis. Sigdestad *et al.* (1969) also reported that the peak in cell synthesis occurred two to three hours prior to the peak of cell division rates.

Using an innovative double labelling technique, Grube *et al.* (1970) have shown that the number of cells entering and leaving the synthetic phase of the cell cycle also shows circadian periodicity. Thus some of the work of Pilgrim *et al.* (1963) could be substantiated. Grube *et al.* (1970) concluded that it was not the rate at which individual cells pass through the phases of the mitotic cycle, but the numbers of cells completing one phase and beginning the next that varies.

Challoner *et al.* (1981) compared autoradiography and liquid scintillation of incorporated [^{3}H]thymidine and found that the latter revealed a circadian rhythm of synthesis in a tissue with a low mitotic rate (rodent kidney). These workers suggested that liquid scintillation was a more sensitive method of detection than autoradiography or mitotic arrest methods and that Pilgrim *et al.* (1963) could have failed to show a circadian rhythm in this tissue for this reason.

Wirth *et al.* (1982) deemed it essential to understand the inherent rhythm in cell division rate when using hamster ear epidermis or sebaceous glands to study cell kinetics. Similarly, for the wool follicle it is important to know if there is any fluctuation in activity throughout the day, since this could very easily bias results of experiments where one sample is taken several hours after another. In the same manner as seasonal changes in the rate of wool growth may

influence the result of longer term hormonal treatments, a change in the mitotic activity of a follicle over a short term may not be entirely due to the influence of a treatment. Above are some examples of such effects in tissues of rodents, and there are equivalent examples in the tissues of other animals, including man. To follow are some examples of hormonal changes which accompany these changes. Man is the species used to illustrate these changes rather than rodents, purely because a more concise and clear review was available for the latter. (It may be important to record the obvious here, that despite domestication, the laboratory rat remains a relatively nocturnal animal and becomes active out of phase with man and the sheep).

2.3. Circadian hormone and substrate concentration profiles in humans.

There is a large resource of literature on circadian rhythms in humans, and this section provides a brief summary of the work of a few authors who have provided a relatively concise editorial review of circadian cycles in plasma hormone and substrate concentrations. The salient points have been related here to show some of the currently accepted models of cycles in the human, as it is possible that patterns of this nature could also occur in sheep.

From a review by Oswald (1978), it appears that growth hormone and prolactin are regulated by the sleep and wakeful cycles in humans. If sleep is delayed, the rise in growth hormone which accompanies sleep is also delayed. Thyroid stimulating hormone has also been shown to be higher at night (Oswald, 1978), but whether this was coincidental or governed by sleep patterns had not been demonstrated at that time. Plasma amino acid concentrations have been shown to be elevated at night, but a change in the sleep pattern relative to the light-dark cycle can cause a change so that the peak plasma levels coincide with sleep as do the levels of growth hormone and prolactin.

Åkerstedt and Levi (1978) from the same editorial review addressed the question of circadian rhythms in cortisol levels along with the levels of adrenaline and noradrenaline in humans. **They also drew attention to the fact that plasma levels are a result of an interplay of secretion into the plasma and excretion which removes hormones from the circulation.** Unlike the compounds considered by Oswald (1978) (above),

corticosteroids appear to rise shortly before an individual would normally awaken, whether this time had been preceded by sleep or not and thus appeared to be regulated in a purely circadian manner in humans. It also appeared to be inflexible to changes in the activity cycle such as shift changes in shift workers, or unresponsive to rapid changes in the light dark cycle as in the case of 'jet-lag'.

Åkerstedt and Levi (1978) also made reference to the difficulties associated with obtaining around the clock estimates of plasma catecholamines and thus gave examples where excreted levels were used as an indicator. Generally, low levels of adrenaline excretion were associated with sleep and very high levels were observed in the period immediately preceding the next sleep. In addition, deprivation of sleep did not change the normal pattern of excretion even when environmental cues were removed and this, along with slow changes in the cycle to perturbations in activity of shift workers led to the conclusion that plasma adrenaline levels were generally circadian in nature.

In sharp contrast to adrenaline, noradrenaline has been found to adjust quickly to changes in the environmental stimuli such as constant lighting, food intake, wakefulness and activity (Åkerstedt and Levi, 1978). Unlike the corticosteroids and adrenaline, changes in shift-work or of time zones cause a rapid adjustment in noradrenaline excretion levels. Thus Åkerstedt and Levi (1978) concluded that noradrenaline levels are regulated by external stimuli, but cortisol and adrenaline are regulated by internal mechanisms which are entrained by the environment.

Schlierf (1978) reviewed the circadian variations of blood sugar and plasma amino acid levels. It seems that dietary intake has a major influence over blood sugar levels whereas amino acids exhibit changes subject to intake, mobilisation, metabolism and excretion, the latter three of which are dependent on the levels of various hormones. Thus blood sugar is controlled by an activity or environmental cue (intake) and amino acids tend to be controlled in a circadian manner. Plasma triglyceride also shows a circadian variation with peak concentrations during the night, and in concert with this there are fluctuations in chylomicrons and very low density lipoproteins, but not levels of low density lipoproteins or cholesterol. In this review, Schlierf (1978) also commented that since the diet normally contains a mix of carbohydrate, fat and

protein and this varies at different meals and on different days, so too the plasma levels of substrate molecules vary. Another important point made was that pathways which interconvert the different groups, can be utilised e.g. triglyceride can substitute for sugar as an energy source.

In brief, circadian variations in hormone and substrate levels have been extensively investigated for humans, and our understanding is quite refined. There are some which can be readily changed in response to shifts in environmental cues and others which are slow to change. Some are controlled by activity or sleep, and yet others by feed intake or light-dark cycles. Our understanding of these hormones in sheep is nowhere near as extensive. There is the added complication that sheep are ruminants and digestion and absorption differ from the monogastric human and thus substrate levels and the hormones which influence them are related in a different manner. The following section (Section 2.4) reviews some of the literature available for circadian variations in the plasma components of sheep.

2.4. Circadian fluctuations evident in plasma hormones of sheep.

The hormones reviewed in this section have previously been shown to have some effect on wool growth rates, (Chapter One) and the text to follow is biased toward growth hormone and cortisol since they are favoured in the literature. There is either little interest in understanding circadian changes in the levels of other hormones, or accurate assay techniques and suitable collection methods have not yet been devised. A recent exception is that of melatonin, which is intimately related to the cycle of daylight, yet appears to have little effect on wool growth (Section 1.2.2(f).).

2.4.1. Thyroid Hormone.

Thyroid hormone is believed to facilitate rather than regulate wool growth (Hynd, 1989b), and it has been neglected in this review because if seasonal fluctuations in thyroxine are inadequate to account for the annual rhythm in wool growth rate (Maddocks *et al.*, 1985), then by analogy it is doubtful that circadian changes in the level of thyroxine would affect wool growth either.

2.4.2. Catecholamines.

McDowell (1983) observed that although a number of studies had been undertaken to determine the effect of catecholamines infused into the animal, none had been undertaken to measure the normal peripheral levels of these hormones. A major problem is that the act of blood sampling itself is sufficient to cause the release of adrenaline, a point made by Åkerstedt and Levi (1978) with respect to humans.

However, circumstantial evidence is available which gives a broad outline of some likely changes. Under conditions of constant environmental temperature, sheep aware that a meal was imminent had elevated levels of a substance with "adrenaline-like activity" (B.P. Setchell, personal communication). The term "adrenaline-like activity" is used, because the actual observations were a decline of the surface temperature of the ear of the sheep, used by Setchell and Waites (1962), a phenomenon which could be reproduced by the infusion of adrenaline.

Tindal *et al.* (1985) provide another source of circumstantial evidence, which is reiterated below in the section related to levels of growth hormone. The inflation of a balloon in the rumen causes a rapid decline in growth hormone levels in goats, a response regarded as "neurally-effected". Prior to this Bassett (1971) and Hertelendy *et al.* (1969) had shown that adrenaline infusion depressed plasma growth hormone concentrations in sheep. Thus in sheep fed once daily, we might expect that adrenaline levels would be elevated in the morning prior to feeding and decline as the feed is consumed.

Plasma noradrenaline levels could be expected to vary as environmental temperature varies. (It is difficult to say whether the levels would vary under conditions of controlled temperature; this point was not made clear by the authors cited for the observations in humans (Åkerstedt and Levi, (1978) in Section 2.3. above.). In summary, both noradrenaline and adrenaline might be expected to vary throughout the day with respect to activity cycles, as in the human (Section 2.3 above) and in addition could be influenced by rumination. It is also important to observe the cautionary note of Esler (1982), that plasma noradrenaline levels are not an accurate indicator of the level of activity of the sympathetic nervous system.

2.4.3. Growth Hormone.

Early work had shown that plasma growth hormone levels declined after feeding (Wallace and Bassett, 1970; Bassett, 1972; Lofgren and Warner, 1972), and became elevated during periods of fasting (Driver *et al.*, 1979). Bassett (1974a and b) demonstrated that feed intake *per se* produced a rapid fall in plasma growth hormone and the fall was not related to changes in either plasma glucose or amino acid concentrations. Of greatest significance to the present report, Bassett (1974b) showed that wethers fed once daily exhibited circadian fluctuations in growth hormone (and insulin). In addition, these were found to be dependent on the level of feed intake, with mean growth hormone levels being depressed by higher intake. Using the inflation of a balloon in the rumen, Tindal *et al.* (1985) were able to show that this effect was due to rumen distension.

Driver and Forbes (1981) showed that short term fasting (ten hours) elevated growth hormone levels, and were also able to show that growth hormone levels were actually falling before the intake of feed under conditions of regular daily feeding. Tindal *et al.* (1985) similarly found that growth hormone levels decreased in goats anticipating a feed.

Thus it seems that the level of neural activity influences plasma growth hormone levels, whether due to the sensation of rumen filling or anticipation of a daily feed. Levels are highest during periods of fasting, and, in sheep given a single morning feed, are highest early in the morning before feeding and remain low throughout the afternoon and night to eventually rise to pre-feeding levels the following morning. In view of the depressant effects of growth hormone on wool growth, we would expect that wool growth rates would be depressed in the morning before feeding and accelerate during the morning and afternoon.

2.4.4. Prolactin.

Davis and Borger (1974) demonstrated a circadian rhythm in the plasma levels of prolactin (and an accompanying rhythm in growth hormone levels) in ovariectomised ewes, with a peak at

around 1800 to 2200 hours. Little information was provided on the diet of these animals, however the sheep were apparently fed twice daily (at 0800 and between 1500 and 1600 hours). Reference to the feeding regime was made in a figure in the Results section, and at one point in the Discussion section, but not in the Materials and Methods section of this paper and little detail can be extracted from the paper on this aspect. Davis and Borger (1974) concluded that the circadian rhythm in prolactin and growth hormone concentration was not tied to time of feeding.

Barrell and Lapwood (1978) observed a rise in plasma prolactin levels in rams shortly after lights out at 1930 hours, when the animals were maintained under conditions of 14.5 hours of light.

Kennaway *et al.* (1981) examined circadian variation of plasma prolactin in rams at four times during one year. This experiment revealed that the circadian secretory pattern was dependent on season. Plasma levels were highly variable, but in general peak levels were observed between 1030 and 1330 hours and also between 2200 and 2300 hours during spring. During summer the peak occurred at night, whereas in autumn it was measured between 1330 and 1730 hours, and in winter there was no significant change throughout the day.

Leshin and Jackson (1987) investigated this phenomenon further and discovered that prolactin was significantly higher in ewes maintained under conditions of sixteen hours light, than in a similar group kept under eight hours of light. Both groups were housed in controlled environment rooms under similar conditions of temperature.

In summary, the light-dark cycle has a major influence on plasma prolactin levels in sheep. However, the timing and magnitude of the peak level is dependent on the conditions imposed.

2.4.5. Cortisol.

In the literature there is some conflict over whether circadian fluctuations of plasma cortisol levels exist in sheep. The balance of the evidence given below suggests there is.

McNatty *et al.* (1972) showed that sheep confined to indoor pens and fed *ad libitum*, exhibited elevated cortisol levels for up to one month. The level was calculated as the mean for three ewes, from which blood was sampled seven times, during every seventh day. After one month, the ewes appeared to become accustomed to the environment, and it was shown that although cortisol release is pulsatile, there was a definite peak in cortisol levels at 0930 hours and that peak plasma concentrations were between 18 and 20 ng/ml. Another three ewes kept under constant lighting did not exhibit a peak in the morning but showed nine or ten smaller peaks throughout the day. After only seven days of confinement three other ewes exhibited cortisol levels between 10 and 18 ng/ml and more than eighteen peaks in concentration, a sign that the animals were suffering some form of stress. Although constant lighting would disrupt circadian phenomena, from this work alone, it is obvious that animals should always be provided with adequate time to "settle down" prior to the commencement of experimental procedures.

In a study by Bassett (1974b) (outlined above for growth hormone) no circadian rhythm in plasma corticosteroid concentration was observed, although the animals were accustomed to the diet and venipuncture sampling for one month, as were the animals used by McNatty *et al.* (1972). In contrast to the experiments of McNatty *et al.* (1972) lights were switched on every 45 minutes to take night time samples, a factor which may have influenced the cortisol levels.

Holley *et al.* (1975) were able to show a circadian rhythm in cortisol levels, however they also presented evidence to show that time of feeding modified this rhythm. In contrast to McNatty *et al.* (1972) the animals were fed at 1600 hours only, and a peak in cortisol levels was evident at this time. A peak was also observed at 0400 hours before the lights were switched on at 0600 hours, suggesting that cortisol levels are also controlled by lighting.

Kennaway *et al.* (1978) observed no circadian rhythm in plasma cortisol levels of wethers that were fed twice daily. To minimise disturbance of the sheep, an automated blood sampling device was used in this experiment, so that no person approached the sheep other than to feed the animals and clean the room. All plasma cortisol measurements were below 6 ng/ml in this

experiment, which suggests that other workers may have observed artificially high cortisol levels due to frequent disturbance of the animals. From recent findings it is likely that sampling techniques may cause some change in plasma cortisol level (Fell and Shutt, 1985; Parrott *et al.*, 1988; Cooper *et al.*, 1989). Kennaway *et al.* (1978) suggested that this could be the origin of the observed circadian rhythm, such that the act of sampling and related release of cortisol produced an artificial rhythm. An alternative explanation is that the rhythm observed by other workers (McNatty *et al.*, 1972; Holley *et al.*, 1975) was created by the daily feeding routine. In the experiments of Kennaway *et al.* (1978) the sheep were fed at 1100 hours and again at 1600 hours, with lights on at 0700 and off at 2100 hours. This may have suppressed the rhythm by separating the environmental stimuli when compared with a once daily feed shortly after "lights on" in the experiments of McNatty *et al.* (1972) and Holley *et al.* (1975).

Fulkerson and Tang (1979) examined the case further, and found that sheep fed *ad libitum* exhibit a circadian rhythm in plasma cortisol. In addition an ultradian rhythm accompanied the circadian rhythm, a series of peaks and troughs suggesting a pulsatile release of cortisol. With "lights off" at 1825 hours and on again at 0625 hours, a peak was evident at around midnight and a trough at 1600 hours. At least one sheep showed evidence of a further peak at feeding time, but since *ad libitum* feeding was imposed, and no observations were made of actual meal intake times, it is difficult to say whether the steroid levels are related to feeding behaviour.

Finally, Foldes *et al.* (1985) measured a circadian change in cortisol levels in wethers fed a slightly restricted diet (600 g lucerne chaff and oats in a 60:40 mix). No mention of the time of feeding was made in this paper but cortisol levels peaked at around 2100 hours.

2.4.6. Melatonin.

Although much of the relevant literature has involved the study of photoperiodic control of reproductive characteristics (as noted previously in Section 1.1.6.3.), essentially, melatonin is elevated during the hours of darkness and suppressed during the daylight hours (Kennaway *et al.*, 1981, 1987). Work specifically related to wool growth is of greater relevance to the present topic, and as shown above (Section 1.2.2(f).), melatonin appears to have no effect on

wool growth over long-term treatments. In the work of Foldes *et al.* (1985), the activity of an enzyme in the pathway of conversion of serotonin to melatonin, was found to fluctuate in concert with plasma cortisol mentioned in the previous section (More precisely, *ln*(serotonin N-acetyltransferase), the natural logarithm of serotonin N-acetyltransferase activity, was highest at night). (This paper is of further interest as it provides a convenient point to connect this section with the next: Depilation force, or the force required to remove a staple of wool of a given cross sectional area from the skin of an animal, was shown to vary throughout the day and will be discussed further in Section 2.5. below.). Cycles of both cortisol and serotonin N-acetyltransferase were found to fluctuate out of phase with depilation force. In view of the apparent lack of effects of melatonin on wool growth, and the inhibitory effects of cortisol, the rhythm in serotonin N-acetyltransferase is possibly less important to the circadian rhythm in depilation force.

2.4.7. Summary of circadian fluctuations in hormone levels.

As a concluding remark to Section 2.4, it seems that there are circadian fluctuations in the plasma levels of hormones which, from Chapter One, have the capacity to affect wool growth rates. Evidence for circadian changes in the levels of thyroid hormones and catecholamines is scant in sheep and it is necessary to draw on some circumstantial evidence for catecholamines at least. There is strong evidence to suggest that there are circadian fluctuations in the levels of growth hormone, prolactin and cortisol. The level of nutrition and the time of feeding have some effect on these fluctuations, however the picture at present is not as clear as in the human. The light-dark cycle has an influence on the circadian fluctuations at least for cortisol and prolactin in sheep. These fluctuations are sufficiently great in magnitude to suggest the physiological feasibility of wool growth rates being influenced throughout the day. The following section provides some evidence for the existence of a circadian rhythm in wool growth rates and cell division rates in wool follicles.

2.5. Circadian fluctuations in wool follicle activity.

From the previous section, it would seem that the rate of cell division in more rapidly dividing tissues (e.g. the anagen hair follicle or the jejunal crypt) does not fluctuate, whereas the comparatively slowly dividing epithelial tissues demonstrate a cyclic change in mitotic activity in rodents throughout the day. Whether the very slowly dividing tissues exhibit periodicity of mitosis is not evident since the rate of mitosis itself is difficult to determine. Challoner *et al.* (1981) showed that a circadian rhythm in DNA synthesis exists in the cells of the rodent kidney and, as mentioned before, concluded that other techniques may not be sensitive enough to detect such a rhythm.

It is yet to be completely determined whether the wool follicle should belong to the group of rapidly or slowly dividing tissues. In this manner, wool follicles could quite possibly be subject to a different form of control than hair follicles, having escaped from the regulation which causes annual shedding as observations on a less microscopic/ biochemical scale would suggest. (In fact the distinction between hair and wool itself is difficult to determine. Some authors have referred to fibres less prominent than the guard hairs of goats (*Capra hircus*) as "wool" (Andersson *et al.*, 1963). Some very early workers referred to the "hair" and "wool" fibres within the one fleece of *Ovis aries* and *O. ammon* (Crew, 1921) and of *O. vignei*, *O. musimon*, *O. montana*, *O. orientalis* and *O. ammon sp.* (Crew and Blyth, 1923). Fraser and Short (1960) reviewed some work on the "halo-hairs" or "birthcoat" of lambs and more recent texts (Chapman and Ward, 1979; Orwin, 1979) have described fibres of an intermediate kind as "medullated wool fibres".

Throughout the present work the word "wool" refers to the fibre grown from the fleece-growing portions of *O. aries*. Medullation of the fibre was present in four animals described in the following experiments undertaken by the present author (three Tukidales and one Suffolk.). (It is possible that a low incidence of medullation was present in the fleeces of other animals, e.g. Corriedales and strong woolled Merinos; however such medullation was not obvious.)

Foldes *et al.* (1985) have demonstrated that different amounts of force are required to pull wool from the skin of sheep at different times of the day. More specifically the natural logarithm of the depilation force, [*ln*(depilation force)], exhibited a circadian rhythm, under conditions of natural daylight and temperature. Depilation force was defined as the force required to pull a staple of wool of a certain cross sectional area from the skin of an animal. The lowest values were apparent at around 1800 hours and peak values at around 0900 hours in six Merino wethers which experienced darkness from 2100 hours to 0700 hours. In ovariectomised and entire Border Leicester ewes which experienced darkness from 1700 hours to 0700 hours the lowest *ln*(depilation force) values were apparent at around 0100 hours and the highest values at around 1200 hours. As mentioned earlier, it was not reported when the sheep were actually fed, but they were offered 600g of lucerne and oats (Section 2.4.5. above).

It is interesting that Foldes *et al.* (1985) removed one ewe which had not been ovariectomised from the analysis because it exhibited "abnormally high" values for the *ln*(depilation force). Kenshalo (1966) has shown that the 'cool threshold' of the skin of female humans varies during the menstrual cycle, and that this was related to the fluctuations of progesterone throughout the cycle. It is possible that a similar relationship may occur in the skin of the ewe during the oestrus cycle and this could help explain the "abnormally high" values of *ln*(depilation force) observed for this ewe. Since progesterone is a steroid, this provides more evidence to suggest that the effects of steroids on the skin are important variables to consider.

Preliminary experiments in these laboratories have suggested that wool follicles exhibit a circadian fluctuation in the rate of mitosis (Hynd *et al.*, 1986; Scobie and Hynd, 1987). A peak in cell division rate was observed in skin samples taken at 2300 hours from a Merino wether sampled every three hours (Hynd *et al.*, 1986). In a Suffolk and a Lincoln ram fed once daily at 0900 hours, a peak in cell division rate was detected at 2100 hours (Scobie and Hynd, 1987). The animals were housed in a controlled environment room, in which the temperature was maintained at 22 ± 4 °C and the lights were switched on at 0700 hours and off again at 2015 hours.

In the work of Scobie and Hynd (1987) it was also noted that the circadian fluctuation in mitotic rate may have been influenced by the level of nutrition. When the same Suffolk and Lincoln rams were offered a smaller quantity of lower quality feed, the peak rate of cell division was less pronounced (Scobie and Hynd, 1987). Under the conditions of restricted nutrition, the peak in cell division rate was measured at around 1800 hours, shortly before the lights were switched off.

2.6. Summary of evidence implicating circadian fluctuations in the rate of cell division in wool follicles.

There is a large body of evidence for circadian fluctuations in cell division rates in some tissues of rodents and also in the plasma levels of hormones and substrates in humans. There is evidence to suggest that similar circadian changes occur in the rate of cell division in wool follicles and also in the plasma levels of hormones which may in some way regulate the rates of cell division in wool follicles.

It is difficult to reconcile some of the observed changes in hormone levels with those measured in cell division rates. The peak cell division rate and the highest levels of some of the hormones which inhibit wool growth rate, occur at about the same time. For example the fluctuations in the mean level of plasma cortisol measured by Foldes *et al.* (1985) were around 15 ng/ml at the peak (2100 hours) and about 8 ng/ml at the trough (1300 hours). The peak level of cortisol lay in the range reported to inhibit wool growth rate (Chapman and Bassett, 1970), but was almost coincident with peak cell division rates (2300 hours) determined by Scobie and Hynd (1987). A circadian rhythm in the rate of DNA synthesis, out of phase with the rhythm in the rate of mitosis was demonstrated in mouse tissues (Pilgrim *et al.*, 1963). It may therefore be possible that the hormones exert their influence at the level of synthesis, and the lag between synthesis and division leads to the coincident peaks in inhibitory hormone levels and cell division.

It is easier to reconcile the rhythm in depilation force measurements with the circadian rhythm of plasma cortisol (Foldes *et al.*, 1985), and it remains possible that this hormone affects wool growth at the level of keratin synthesis rather than cell division. As shown in Chapter One,

wool growth can be considered as a compartmental process, where cell division is followed by keratin synthesis. A hormone could therefore exert its effect in one compartment more rapidly or extensively than another.

2.7. Reasons for the chosen experimental approach.

Hynd *et al.* (1986) observed a circadian rhythm in the rate of cell division in the follicles of a Merino. In contrast, Scobie and Hynd (1987) found no such rhythm in this breed, however a decisive rhythm was detected in follicles sampled from Suffolk and Lincoln sheep. It was therefore of interest to reassess the Merino breed for circadian periodicity of wool follicle mitotic activity.

Although the Suffolk breed is not generally regarded as a wool sheep, the rate of wool production per individual follicle of this animal was high when compared with the Merino, the difference in density accounting for the difference in total output. To examine other breeds which could be expected to demonstrate distinct differences in the total rate of wool follicle activity was perceived as important. For these reasons, Merino, Corriedale and Tukidale sheep were chosen as they could be expected to respectively exhibit low, intermediate and high relative rates of follicle activity.

Although a range of wool growth rates was one aim of this approach, it must be noted that the primary objective of this experiment was to demonstrate circadian variations in the mitotic rate of wool follicles from individual animals. That is, rate changes from one time period to the next in skin samples taken from the one animal was the major point of interest. The following experiment was designed to provide further evidence for circadian fluctuations in cell division rates in wool follicles, and to determine whether the rate is controlled by the time of feeding or the time of day. Three different breeds were used to determine how the circadian fluctuations vary between breeds and also between animals with different types of "wool" and rates of wool growth.

The statistical approach adopted in this experiment was to collect a large number of samples from a few individuals, based on the evidence of Scobie and Hynd (1987). The reasons for this are clearly outlined in Appendix Two. The use of time series analysis on this type of data was perceived as the most efficient way of determining whether a circadian rhythm exists in the rate of cell division in the wool follicle.

The present experiment was designed to test the following hypotheses:

Wool follicle mitotic activity demonstrates a definite circadian periodicity.

This circadian periodicity is related to the dark and light cycle.

This circadian periodicity is related to the time of feeding.

This circadian periodicity varies between breeds.

2.8. Experimental Procedure.

2.8(a). General.

Six, fifteen month old wethers were housed in a controlled environment room. The lights were switched on at 0700 hours and off again at 1715 hours, and the temperature maintained at a minimum of 15.5 ± 1.4°C and a maximum of 18.7 ± 1.5°C. The six sheep consisted of two Merinos (38 kg), two Corriedales (37 kg) and two Tukidales (43 kg).

2.8(b). Diet and Feeding protocol.

One animal from each breed was fed between 0800 and 1000 hours and the other between 2000 and 2200 hours. The daily ration consisted of 800 g of pellets containing lucerne chaff and field beans (*Vicia faba*)in a 60:40 mix. (88% digestible dry matter, 18% crude protein).

2.8(c). Skin sampling protocol.

After seven weeks of feeding the above ration, twelve skin biopsies were removed from each animal over a single twenty four hour period. Skin samples were taken from midside sites as

shown in Figure 2.2 every two hours according to the method of Hynd *et al.* (1986). The following modifications were made to adapt the method to this experiment. Intradermal colchicine was injected two hours before sampling rather than three as used by Hynd *et al.* (1986). The dose of colchicine used to achieve arrest was ascertained in a separate experiment (See Appendix One), the Merinos received 10 μg of colchicine, and the Corriedales and Tukidales 50 μg. The first colchicine injections were made at 0800 hours and sampling began at 1000 hours.

Tissue was stained using the haematoxylin and eosin method outlined by Hynd *et al.* (1986). This procedure produces darkly-stained nuclei which have been arrested at metaphase by colchicine, and can readily be counted under light microscopy using a 40 x objective. Random counts were made as per the method of Schinckel (1962).

2.8(d). Statistical analysis.

For each separate sheep, the average number of mitoses per follicle bulb, corresponding to various times of the day, was tested for a pattern using the turning points method of Kendall (1976).

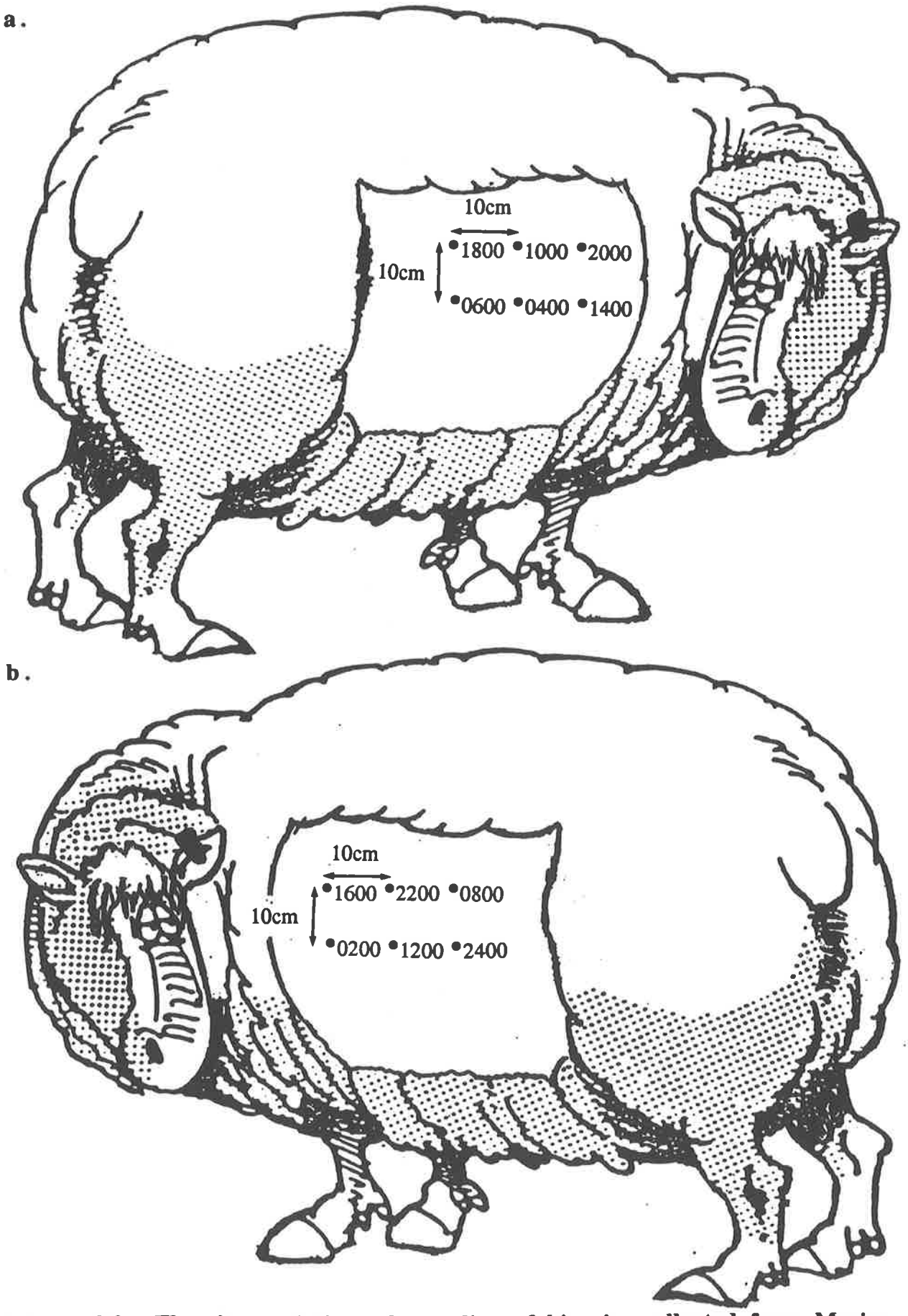


Figure 2.2. The sites and time of sampling of biopsies collected from Merino, Tukidale and Corriedale wethers (a) on the right midside and (b) on the left midside. Adapted from Ball (1979).

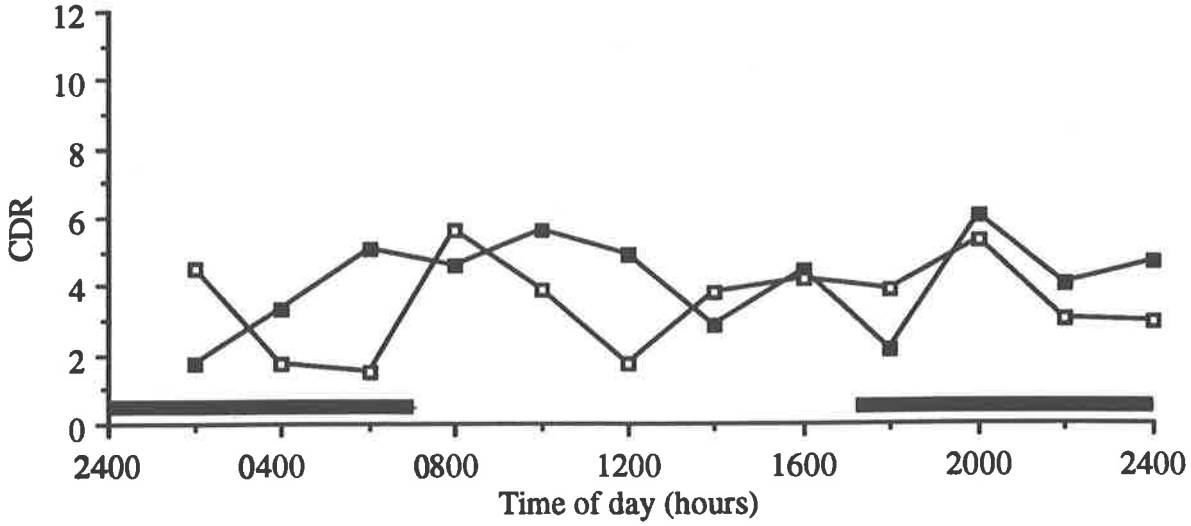
2.9. Results.

The average number of mitoses arrested per 10 μm thick section of a follicle bulb over each two hour period is plotted against the time of day for sheep one to six in Figures 2.3a, 2.3b and 2.3c. The two different feeding periods are also indicated on the time axis, as is the period of darkness.

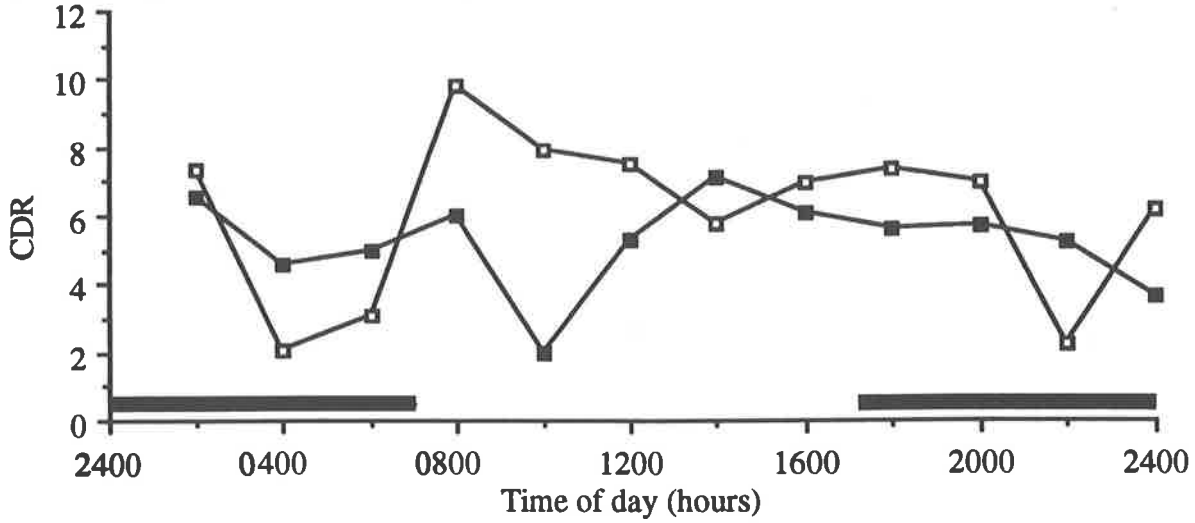
The most important thing to note from Figures 2.3a to c is that the rate of cell division in the wool follicles of these animals was not constant. The rate of cell division in the follicle bulbs of all sheep fluctuated widely over the twenty-four hour period. Up to four and six fold changes were evident in all animals.

However, there was no clear pattern to the changes in cell division rate. The turning points method revealed, that for no individual was the variation other than random. Although statistical analysis revealed no significant effect of the time of day, or time of feeding on cell division, it is interesting to note that there was a reduction in cell division rate in the morning, shortly before "lights on" in all of the sheep which exhibit fast wool growth rates (Corriedales and Tukidales). Of these four animals, those fed at night also exhibited a reduction in the rate of cell division, at around the time of the evening feeding.

a. Merino wethers. (For each point, s.e.m. < 0.2, n > 250 follicles).



b. Corriedale wethers. (For each point, s.e.m. < 0.2, n > 250 follicles).



c. Tukidale wethers. (For each point, s.e.m. < 0.3, n > 250 follicles).

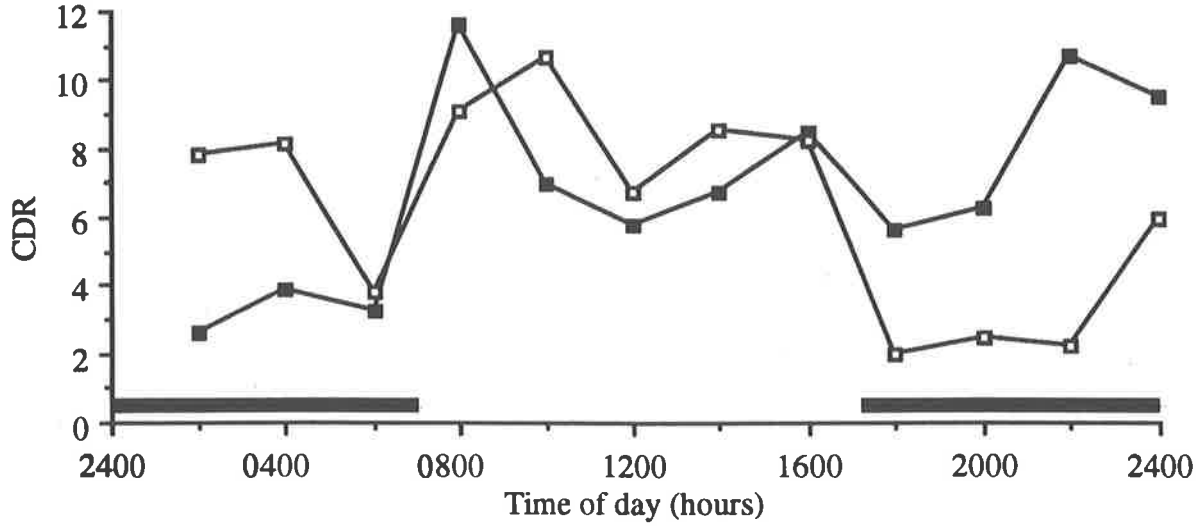


Figure 2.3. Cell division rate plotted against time of day for day-fed (■) and night-fed (▫) animals. The period of darkness is indicated by the horizontal black bars.

2.10. Discussion.

In general Corriedale and Tukidale sheep exhibited faster cell division rates than those observed in Merino animals, hence the objective of selecting sheep that were expected to demonstrate more rapid rates of cell division was achieved. (Note that the present rates were calculated as the mean, and those of Scobie and Hynd (1987) as the median and a direct comparison of the values is not valid). The present results confirm those of Hynd *et al.* (1986) and Scobie and Hynd (1987), in that cell division rate varied widely throughout the twenty-four hour period of sampling. Similarly, those animals which exhibited rapid rates of cell division demonstrated the greatest fluctuations, and there was a suggestion that mitotic activity was lowered at the time these animals were accustomed to being disturbed. Unlike the previous results, those obtained presently indicate no clear circadian rhythm in mitotic rate. Certainly for the Merino animals, the fluctuations appeared random with respect to the time of day, or time after feeding. Some possible explanations for the discrepancy are pursued here.

It is possible that the random fluctuations observed in the present experiment were an artifact of the sampling regime. The night time samples were taken with the aid of a dim red light, and it was considered unlikely that disturbed lighting patterns were the cause of the discrepancy. However, some animals became notably excited and aggressive during the course of skin sampling. The work of Fell and Shutt (1985) and Parrott *et al.* (1988) showed that restraint and handling can cause elevated secretion of cortisol. Excitement and distress would undoubtedly cause elevated secretion of adrenaline as well. It was therefore considered quite possible that a hormonal response to stress could have influenced the rate of cell division determined in the present experiment and masked any circadian variation normally evident.

It remains possible that the results of all three studies could be an artifact of the respective sampling regimen. Just as the erratic fluctuations observed here may have been biased by the sampling techniques, it is possible that those of Hynd *et al.* (1986) and Scobie and Hynd (1987) may have been caused by the experimental conditions imposed.

The Lincoln and Suffolk rams studied in the experiments of Scobie and Hynd (1987) became nervous, excited and eventually aggressive during the course of the sampling (unpublished observations). These two animals showed the clearest circadian rhythm in cell division rate. Foldes *et al.* (1985) have previously demonstrated a circadian rhythm in *ln*(depilation force) and similar changes in the rate of cortisol secretion. In view of the results obtained by Foldes *et al.* (1985), it became of interest to study the levels of cortisol in plasma samples which had been collected during the skin sampling period at the high level of nutrition in the experiments of Scobie and Hynd (1987). Plasma samples had been obtained every three hours, concurrently with all colchicine injections. An additional blood sample had been taken, coincident with the final skin biopsy event at the conclusion of the sampling period. This provided nine blood samples for each of the animals, which had been stored at -20°C.

A cortisol assay kit was obtained for the purpose of analysing plasma cortisol levels (Amersham Radiochemicals company, Amerlex Cortisol RIA kit.). Triplicates of each plasma sample were assayed according to the method detailed in the kit, and the average cortisol level determined in ng/ml. In only two of the twenty-seven samples was the cortisol concentration greater than 10 ng/ml, and all were within the range of levels discovered in plasma samples from unstressed animals by Bassett and Hinks (1969). The two plasma samples which did show a high cortisol concentration were the first sample taken from the Suffolk and Lincoln rams. Although cortisol is released in a pulsatile manner (McNatty *et al.*, 1972; Holley *et al.*, 1975; Fulkerson and Tang, 1979) and three-hourly samples are inadequate to reveal true cortisol levels, it was considered possible that the observed changes in mitotic rate in these animals (Scobie and Hynd, 1987) could constitute a response to the high initial plasma cortisol level. This is pursued further in the following chapter.

Colchicine causes the arrest of cell division at metaphase by stoichiometrically binding to tubulin polymers (Borisy and Taylor, 1967a and b). If the rate of cell division does exhibit a circadian rhythm, the rate of tubulin polymer formation would also be expected to vary throughout the day. It is therefore possible that the dose rate determined at one time of day may be insufficient to produce complete arrest at another, due to dilution of the drug by higher rates of cell division. Schlierf (1978) pointed out that Mayersbach (1976) had shown the same dose

of radiation or drugs could cause between 0 and 100% mortality in rats depending on the time of day they were administered. This deserves special consideration, since the currently observed changes in mitotic rate were of the order of six-fold. Although the required dose of colchicine used in the present experiment was determined in similar animals, subsequent experiments have shown that doses of 100 μg are necessary to achieve total arrest in Lincoln ewes (see Appendix One). The 50 μg dose could therefore have been marginally ineffective at some times of the day.

In conclusion, the random nature of the variation in rates of mitosis observed in the present experiment could be due to any or all of the factors outlined above. At this point, determination of the causative mechanisms or zeitgeber involved in these circadian rhythms was postponed. From the preliminary information obtained from the determinations of cortisol, further investigation of the possible effects of stress hormones was considered to be of primary importance. It was considered possible that the discrepancies in the observations could be explained by stress caused by sampling.

The following chapter was designed to assess the effect of cortisol, the major steroidal stress hormone, on cell division rate. The experiments described in Chapter Four were conducted to determine whether the catecholamines adrenaline and noradrenaline might have some short term influence on cell division. Finally, Chapter Five returns to the theme of this chapter to provide further insight into daily fluctuations in cell division rates in wool follicles.

Chapter Three.

The short term effect of cortisol on cell division.

"Stress and nervous tension are now serious social problems in all parts of the Galaxy, and it is in order that this situation should not be in any way exacerbated that the following facts will now be revealed in advance." (Adams, 1986).

3.1. Introduction.

Literature reviewed in Chapter Two revealed that:

1) Cell division rate in wool follicles has been shown to exhibit circadian fluctuations.

2) Plasma levels of many "stress" hormones exhibit circadian fluctuations.

Thus it seemed possible that the fluctuations in the level of hormones which affect wool growth, could potentially be responsible for the observed circadian fluctuations in cell division rate.

The experimental results of Chapter Two confirmed earlier findings of wide variation in cell division rate throughout the day, but showed random fluctuations in cell division rate, rather than a rhythmic pattern. It was considered that this effect may have resulted from the stress associated with sampling and that:

either the normal rhythm was overridden by the effects of stress involved in the sampling procedure.

or there was no rhythm in cell division rate and earlier results were also a product of similar stress-related effects.

In short, it was not possible to completely dismiss the existence of a circadian rhythm in cell division.

Cortisol was one of the hormones reviewed in Chapters one and two and a considerable body of information is available about its effects on wool growth. Coincidentally, in some studies elevated plasma cortisol has been observed during the early hours of the morning (McNatty *et al.*, 1972; Holley *et al.*, 1975; Fulkerson and Tang, 1979), to levels which have produced a reduction in wool growth rate in other work (Chapman and Bassett, 1970). From the literature, it was also evident that under stressful handling procedures cortisol levels often rise. The minimum length of time for cortisol to elicit an effect on wool growth is unclear, with the shortest of experiments showing a reduction of wool growth rate after a treatment period of four days (Wallace, 1979a).

During the present study it was proposed that if the level of cortisol affects the rate of cell division, then an overriding influence of stress superimposed on a normal daily rhythm could explain both the circadian rhythm observed previously and the random fluctuations observed in the experiments reported in Chapter Two. If elevated cortisol levels inhibit cell division within a short period of time (a few hours rather than four days as measured previously) then they may similarly produce a circadian rhythm in cell division rates under unstressed conditions and random fluctuations under stressful conditions.

The work of Chapman and Bassett (1970) was considered very important, since they showed that the effect of cortisol on wool growth rate can be modified by the level of nutrition. Under conditions of *ad libitum* feeding, a moderate increase in cortisol levels can stimulate intake, with a resultant increase in wool growth rate. In the following experiments, feed intake was restricted to remove this confounding variable.

The studies embarked upon in this chapter were designed to determine whether stressful levels of cortisol could reduce cell division rates in wool follicles. The main goal of this work was to ascertain what length of time is necessary for a change in cell division rate to occur. This was of course facilitated by the method outlined in Appendix One and the short term *in vitro* method outlined in Chapter One.

3.2. General Experimental Procedures.

3.2.1. Collection of blood samples.

Blood samples were collected from jugular catheters inserted eight centimetres into the vein. The catheters were constructed of polyvinyl tubing with an internal diameter of 1mm (Dural plastics and engineering, Dural, NSW). Between sampling the catheters were filled with heparinised saline (10 i.u./ml of heparin in 0.9% NaCl). After collection of the blood into heparinised tubes, the tubes were centrifuged immediately and 5 ml of the plasma removed. The plasma was stored at -20°C until assayed for cortisol.

3.2.2. Assay of plasma cortisol.

For all experiments with the exception of Experiment 3e the following assay procedure was used. These assays were kindly carried out by Ron Newman of the CSIRO Division of Animal Production, at Blacktown NSW. The samples assayed for Experiment 3e were few in number, and the technique is included in the experimental procedure specific to that experiment.

Borate buffer (pH 7.6) was prepared using 1.24 g of boric acid and 2.0 g of gelatin per 1000 mls of distilled water. Sodium azide, 0.05% was added to the buffer as an antioxidant. Hydrocortisone (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was made up as a solution (128 µg/ml) and which was serially diluted to produce standards as follows; 0, 1.25, 2.5, ...640 ng/ml which were assayed in triplicate to produce a standard curve.

Aliquots of plasma or standard (20µl) were pipetted into 12 mm x 75 mm glass tubes and borate buffer (380µl) was added. The tubes were covered with foil and heated for 60 min. at 60°C, cooled to room temperature and if necessary stored at 4°C for up to 5 days. Commercially-labelled [^{125}I]-cortisol (50µl) (Amersham Laboratories, White Lion Road, Amersham, Buckinghamshire, England, HP79LL) was added to each tube. Antisera were prepared from a locally-obtained antibody (cortisol Ab "Pig Pen" 12/1/89, CSIRO, Division of Animal Production, PO Box 239, Blacktown, NSW, 2148, Australia) and used at 1:1800.working dilution, 100µl of which was added to each tube. All tubes were mixed on a vortex mixer and incubated overnight at 4°C.

Dextran-coated charcoal was freshly prepared for each assay using dextran T70 (625 mg) and Norit A charcoal (6.25 g), made up to 250 mls in distilled water. This was added to each tube (100µl), the tubes were mixed on a vortex mixer and incubated for 60 min. at 4°C. The tubes were then centrifuged at 2500 rpm at 4°C for 15 min, the supernatant removed and the amount of radioactivity in the charcoal pellet determined by counting on a gamma counter.

Plasma samples were assayed in duplicate and an internal standard included every 100 tubes. Three additional total count tubes (approximately 6000 cpm) and non specific binding tubes

were included in each assay. Based on five assays, the sensitivity of the cortisol radioimmunoassay was 0.8 ng/ml, and the intra- and inter-assay coefficients of variation were 4.4 ± 0.6 % and 5.7 ± 0.9 % respectively.

3.2.3. Adrenalectomy Procedure.

Merino wethers (50 kilograms) were adrenalectomised by ischaemia induced in the adrenal gland. Constriction of the blood vessels supplying the gland was achieved by means of a latex rubber ring (Elastrator ring) commonly used for castration of livestock (Elastrator Australia, 368 Hawthorn Road, Caulfield South, 3162).

Animals were initially anaesthetised using 0.4 ml/kg sodium pentobarbitone (Nembutal, Ciba Geigy Australia Ltd, Kidman Park, 5025, South Australia.) and subsequently maintained under Halothane (ICI Australia Pty Ltd, 1 Nicholson st., Melbourne 3000, Australia). Entry into the viscera was achieved via an incision on the right hand side of the animal. This approach was similar to that of Buck and Bond (1966), who used an incision which extended from the last rib to the tuber coxa, and parallel to the transverse processes of the lumbar vertebra. With some experience, it was found easier to make this incision parallel to the last rib where it meets the vertebral column. Upon reaching the viscera, a rapid search revealed both left and right adrenal glands.

Elastrator pliers, (Elastrator Australia, 368 Hawthorn Road, Caulfield South, 3162) were loaded with an Elastrator ring and subsequently used to load the device shown in Plate 3.1, a modified 20ml syringe (Terumo Australia Pty Ltd, Melbourne, Australia).

Surrounding adipose tissue was cleared from the right adrenal. The plunger of the syringe was adjusted so that roughly 5 ml of space remained in the open end, which was then gently placed over the gland. The plunger was then smoothly and quickly withdrawn a further 5 to 7 ml, the suction from which lifted the gland into the barrel of the syringe. The Elastrator ring was eased from the end of the syringe using the index finger and thumb, to encircle the base of the gland and thus constrict the blood vessels supplying and draining the gland.

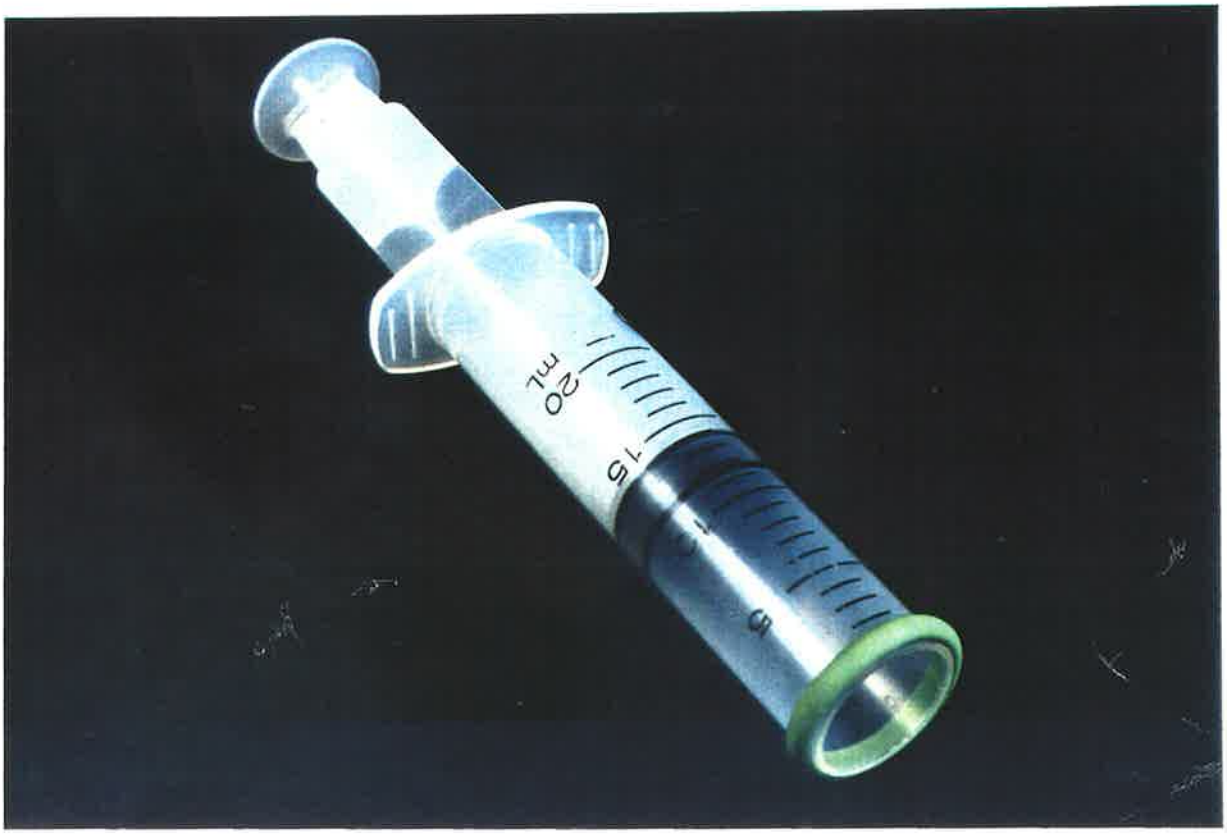


Plate 3.1. A modified disposable syringe 'loaded' with a latex 'Elastrator' ring. This apparatus was used to assist in placing the latex ring around the base of the adrenal gland, to adrenalectomise the animal.

A further Elastrator ring was loaded onto, and held open by the jaws of Elastrator pliers. The left adrenal was located, and the index and middle finger of the surgeons left hand was placed under the gland to lift and support it. The Elastrator pliers were used to place an Elastrator ring over the gland and onto the tips of the surgeons fingers. The Elastrator ring was finally slipped from the ends of the fingers. All instruments were removed from the viscera, and a rapid inspection of both glands undertaken to ensure that each gland was completely surrounded by the latex ring. The wound was then closed and the animal returned to post-operative care.

The loss of blood was kept to a minimum by this technique, and the speed of the operation was enhanced. The success of adrenalectomy by this method was established by measurement of plasma hormone levels after an adrenocorticotrophic hormone challenge (described in Section 3.2.6(a). below), and by post-mortem recovery and histological examination of the tissue associated with the Elastrator ring after the completion of the experiments.

An occasional adrenal was removed by ablation of the gland as per the method of Hofmeyr and Fitzpatrick (1959) although this was not favoured, especially when the proximity of the gland to the posterior vena cava created difficulties. Comparisons under the light microscope were conducted on histological sections prepared from freshly excised glands collected in this manner, and similar tissue destroyed by ischaemia, to study the degradation of the adrenal tissue after occlusion of the blood vessels.

All animals were allowed at least fourteen days of steroid replacement therapy, to recover from surgery before any experiments commenced. For a period of at least seven days leading up to any experiment, cortisol therapy was omitted. (This was flexible, as there were isolated cases which required a single small dose of cortisol to ensure survival of these valuable animals.) Unless specified, the animals were fed 1kg of a pelleted ration with *ad libitum* water and salt (NaCl).

3.2.4. Steroid replacement therapy.

Post-surgically, the animals were maintained by daily administration of 0.05 mg/kg of deoxycorticosterone acetate (Sigma Chemical Co., St. Louis, Missouri, U.S.A.). The dose was administered in 0.4-0.5 ml of an oil vehicle injected subcutaneously into either right or left axilla. Unless otherwise stated, during non-experimental periods the animals were also injected daily with 0.25 mg/kg of cortisol (Hydrocortisone, Sigma Chemical Co., St. Louis, Missouri, U.S.A.) carried in suspension in an oil vehicle.

This regime was based on dose levels published by Ferguson *et al.* (1965). When both cortisol and deoxycorticosterone acetate (DOCA) were administered, these levels were found to satisfactorily maintain adrenalectomised animals for periods of up to six months. When cortisol was omitted prior to any experiment, survival was sometimes compromised but this step was regarded as desirable to develop rapid and profound changes in plasma cortisol to adequately demonstrate changes in wool follicle mitotic rate.

By intensively monitoring the animals and housing them in a controlled environment room during adverse conditions of temperature, it was possible to improve the rate of survival in the absence of cortisol. Four animals were maintained without any cortisol for a total period of twenty days during Experiment 3f. to follow. To maintain these animals, the daily dose of DOCA was lowered from 0.05 mg/kg to 0.04 mg/kg. Regular determination of the packed cell volume (PCV) of these animals enabled successive refinement of the dose rate and the final protocol settled upon was a subcutaneous injection of 0.04 mg/kg of DOCA in 0.5 ml of an oil vehicle injected at 0900 hours.

3.2.5. Monitoring of packed cell volume.

A noticeably lower level of packed cell volume was maintained using the above steroid replacement protocol, than in normal animals maintained side-by-side under identical conditions of feeding and housing. Table 3.1 shows the average packed cell volume observed in six heparinised blood samples (10 ml) collected at intervals of one hour from each of six normal and eight adrenalectomised. The packed cell volume was determined as an aside, to simultaneous experiments described later in this chapter. The averages were established on two consecutive days for each animal under similar plasma cortisol conditions on day one, and different plasma cortisol levels on day two. Clearly, packed cell volume was lower in adrenalectomised animals, however attempts to lower the dose of DOCA further to boost PCV to normal levels were unsuccessful and resulted in severe haemolysis. Analysis of covariance revealed that there was no significant effect of cortisol level on packed cell volume over the range from 0 to 734 ng/ml of plasma (F ratio of 0.33, f probability of 0.724). However, the effect of adrenalectomy and mineralocorticoid maintenance therapy on packed cell volume was highly significant (F ratio of 11.5, f probability of 0.007).

Table 3.1. A comparison of packed cell volume in normal and adrenalectomised animals.

	Day 1	Day 2
Normal Animals	31.95 ± 2.52	30.44 ± 2.71
Adrenalectomised Animals	26.26 ± 3.84	24.29 ± 2.88

As a consequence of the difference in packed cell volume, plasma cortisol levels tend to slightly overestimate the difference between animals when comparing adrenalectomised animals with entire animals in the section to follow. For example, 100 ng of cortisol per ml of plasma would correspond to 70 ng of cortisol per ml of whole blood for an entire animal with a 30% packed cell volume, and 75 ng per ml of whole blood for an adrenalectomised animal with a 25% packed cell volume.

3.2.6(a). Assessment of the success of adrenalectomy.

Adrenalectomised animals were treated with a synthetic ACTH preparation (Tetracosactrin, Synacthen Depot, CIBA-GEIGY Australia Limited, Lane Cove, Sydney, NSW) to establish the efficacy of the new surgical technique. In total, sixteen adrenalectomised sheep (Adx1 to Adx16) were treated with ACTH and four entire animals (Ent1 to Ent4) were treated and sampled in a similar manner to indicate the response which would be observed in a normal animal.

The dosage and blood sampling regime was based on the work of Fell *et al.* (1985), who observed increases in plasma cortisol levels in normal sheep of 300 nmol/l within two hours after treatment using this preparation. A single intramuscular injection (1ml of a solution containing 0.5 mg of Tetracosactrin/ml) of the preparation was administered at 1400 hours. Heparinised blood samples (10ml) were collected at 1300, 1330, 1400, 1420, 1440, 1500, 1600 and 1700 hours.

3.2.6(b). Results of ACTH challenge.

The effects of ACTH stimulation are displayed in Figure 3.1. Cortisol release in the adrenalectomised animals was negligible compared to the stimulation achieved in normal animals housed, fed and treated in the same manner. Cortisol was recorded in six out of one hundred plasma samples collected from fourteen of the adrenalectomised animals, but never exceeded 1.4 ng/ml. The average cortisol level in the unstimulated entire animals sampled

concurrently was ten times higher than plasma cortisol evident in any one sample from the adrenalectomised animals, with two exceptions described below.

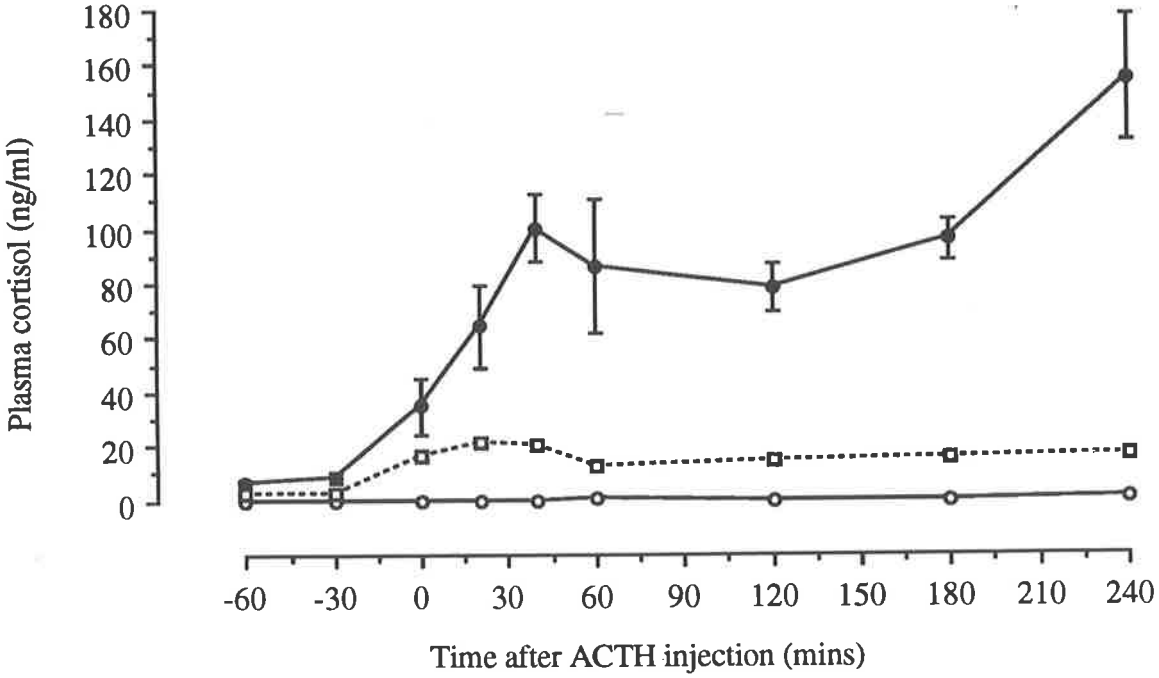


Figure 3.1. The response of adrenalectomised (○) and entire (●) animals to acute stimulation with ACTH, to demonstrate the effectiveness of adrenalectomy by ischaemia. (Adrenalectomised animal Adx15 (□) responded to ACTH).

A small piece of adrenal tissue (0.7g fresh weight) was recovered during the post-mortem examination of one animal, Adx15. This tissue responded to ACTH stimulation, and this animal has been illustrated individually on Figure 3.1. However, cortisol release by this tissue was greatly impaired with respect to the response in animals with a normal-sized and functional gland.

Equally interesting was animal Adx12 which demonstrated measurable levels of cortisol in 20 out of 27 separate plasma samples collected during this and other experiments. The maximum level of cortisol exhibited in any single sample was 7.1 ng/ml, with an average of 1.4 ng/ml. Obviously, there was some source of this cortisol in this animal, however it was not located during an extensive post-mortem examination, nor was it responsive to ACTH.

3.2.6(c). Discussion on the success of the adrenalectomy technique.

Clearly adrenalectomy using this method is a favourable alternative to surgical extirpation. Plasma cortisol was apparent in some animals, however it was only responsive to ACTH in one animal which was readily identified during a post mortem examination. Given that cortisol therapy was withdrawn seven days prior to ACTH challenge, glucocorticoid feedback would be expected to have been minimal, and this should have resulted in elevated endogenous ACTH levels. Assay of the trophic hormone levels could be considered as an alternative method for determining the success of the operation.

A total of thirty animals were adrenalectomised using this method, only two of which died within fourteen days of the operation. One animal never recovered from the anaesthetic and died with an extremely rapid heartbeat. The other animal died of accidental causes not related to surgery.

Other fatalities were recorded after the two week recovery period had elapsed. During periods leading up to experiments when glucocorticoid therapy was omitted, four animals died from heat stress during a period of high ambient temperatures. Following this, other animals were housed in a controlled environment room. Another animal was euthanised when it showed signs of extreme discomfort, subsequently revealed to be due to a blocked urethra. *Ad libitum* salt was provided to all animals in a effort to avoid the formation of urinary calculi, and salt intakes were in general quite high, however the level of intake was not recorded and it is not known whether this animal consumed a great deal of the salt.

Adrenalectomy using this procedure was relatively easy, and the technique was therefore considered to be a breakthrough in the production of animals for the following experiments. The technique will be published as a preferable alternative to previous techniques reviewed by Hecker (1974). By observing the animals at regular (eight hour) intervals and feeding a moderate but restricted diet whilst they were housed in a controlled environment room, it was possible to maintain extremely low levels of plasma cortisol for periods of up to twenty days.

3.3. Investigation of the effects of localised changes in cortisol level.

Studies previously described in Chapter One have shown that locally-administered cortisol or glucocorticoid analogs inhibit wool growth. It was thus of interest to determine whether an effect of cortisol on cellular proliferation can be detected shortly after administration. Experiment 3a was undertaken to test the hypothesis that intradermal injections of cortisol have the potential to reduce cell division at the site of injection, in the short term. Experiment 3b was undertaken to test the hypothesis that DNA synthesis will be reduced by the presence of cortisol in the medium. These two techniques were used simultaneously to provide data which could be qualitatively compared, to indicate whether the hormone would exhibit its action in the presence and absence of blood flow.

3.3.1. Experiment 3a.

The following experiment was designed to determine whether an intradermal injection of cortisol influences cell division rate within a relatively short period of time.

3.3.1(a). Experimental Procedure.

Three adrenalectomised Merino wethers (Adx17, Adx18 and Adx19) were maintained on lucerne chaff (750 grams) for four weeks. At the end of this period, intradermal injections of cortisol were administered into the right midside according to the pattern outlined in Figure 3.2. Four doses of cortisol (0, 10, 100, 1000 μg) were administered using propylene glycol (0.1 ml) as the vehicle. Skin biopsies were removed two, four and six hours later from sheep Adx17, Adx18 and Adx19 respectively. The biopsies were taken two hours after injection with colchicine (50 μg) and treated as per the method of Hynd *et al.* (1986).

3.3.1(b). Results.

The average number of mitoses arrested during a two hour period of colchicine treatment for the intradermal doses of cortisol is shown in Table 3.2. Even at the very highest dose there was no

significant effect of cortisol treatment when compared with the control, with the exceptions of 100 μg at four hours and 1000 μg at six hours. There was some variability between the measured rates of cell division within individual sheep, but no consistent effect with respect to the dose of cortisol across the wide range of doses used. Similarly the results showed little effect of cortisol on cell division rate with respect to the time of exposure to the drug.

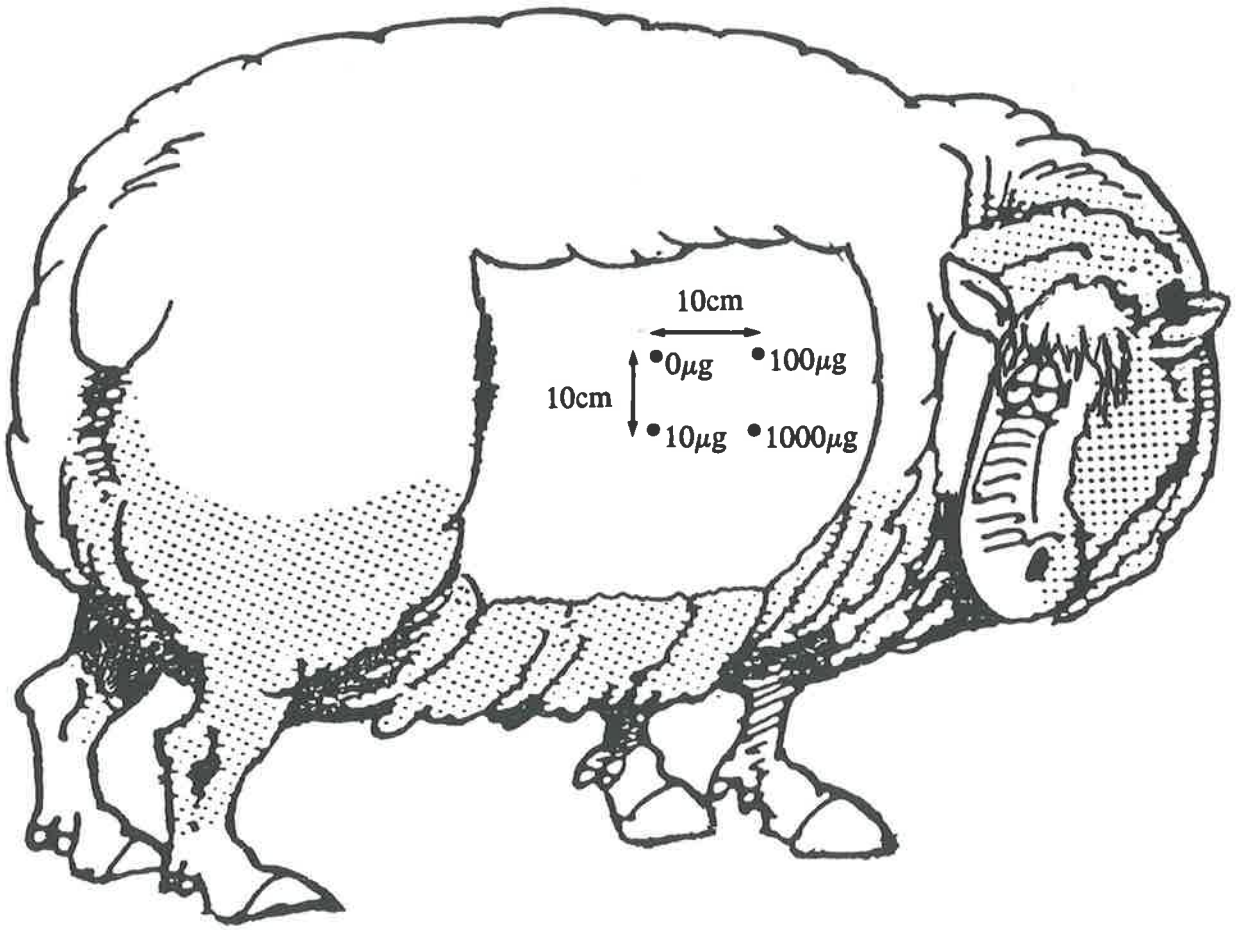


Figure 3.2. The site of injection of intradermal cortisol doses and subsequent biopsies. Adapted from Ball (1979).

Cell division rate was greater in sheep Adx19 than in the other two animals at any given dose rate. These differences were apparent at all dose rates and were much greater than any differences in the measured cell division rate between samples taken from an individual animal. This is an important point, not only in the context of this chapter, but it is for this reason that a difference between cell division rate measured in control and treatment skin biopsies for individual sheep was regarded as more important than the difference between sheep at any treatment level (for this type of experiment).

Table 3.2 Cell division rate in follicle populations treated for two, four and six hour with various intradermal doses of cortisol.

Sheep	Adx17	Adx18	Adx19
Length of cortisol treatment (hours)	2	4	6
Dose of cortisol	Cell division rate		
0µg	4.4	4.0	6.3
10µg	4.2	3.7	5.9
100µg	3.7	4.3	6.1
1000µg	4.2	3.8	5.2

3.3.2. Experiment 3b.

The following experiment was designed in order to determine the effect of various concentrations of cortisol on DNA synthesis in sheep skin *in vitro*.

3.3.2(a). Experimental procedure.

Skin strips 1mm wide and 20 cm long were removed from an adrenalectomised wether (Adx20), by first cutting the skin with a scalpel loaded with two blades spaced 1mm apart, and trimming the strip off with scissors under aseptic conditions. The strips were immediately immersed in a container of warm culture medium (Medium 199, plus Hepes plus Hanks salts) and placed in an incubator (37°C and 5% CO_2). The strips were treated as per the method of Ward and Harris (1976) with the following modifications. Segments of skin, approximately two centimetres in length and one millimetre wide were placed in petri dishes containing 3.2 ml of media. Cortisol was added to replicates of four petri dishes at nine different levels, (0, 1.5, 3, 6, 12, 24, 48, 96, 192 ng/ml). The petri dishes were placed in an incubator at 37°C for four hours. For an additional 30 minutes, the skin strips were placed in fresh media (3.2 ml) containing identical levels of cortisol plus 5µCi of [^{3}H]thymidine (specific activity 9 Ci/mmol) (Amersham Laboratories, White Lion Road, Amersham, Buckinghamshire, England, HP79LL)

At the end of the incubation period the skin strips were removed from the culture dishes and immersed in 10% trichloroacetic acid solution (10ml). After 15 minutes the tissues were taken from the trichloroacetic acid solution and blotted on paper towel to remove excess liquid, weighed and then subjected to three subsequent trichloroacetic acid washes. The tissue samples were then blotted on paper towel to remove excess liquid, the weight of each sample recorded and the samples placed in individual vials containing 0.8 ml of tissue solubilizer (Soluene-350, Packard Instrument Co. Inc. 2200 Warrenville Rd., Downers Grove, Illinois 60515). The vials were capped, placed in a shaking water bath at 60°C and left overnight. After removing the vials and allowing them to cool, 1ml of 1N HCl was added to each and the resulting solution was thoroughly mixed on a vortex mixer. Following this 10 ml of aqueous counting scintillant (ACS 2, Amersham Laboratories, White Lion Road, Amersham, Buckinghamshire, England, HP79LL) was added to each vial, the solutions mixed and left to stand for 48 hours in a dark cupboard. The incorporation of [^{3}H]thymidine was determined by counting on a beta counter (RackBeta, LKB, Wallac, Turku, Finland). Finally the incorporation of radioactivity per mg of wet tissue was calculated as:

$$\text{Incorporation of radioactivity} = \frac{\text{counts per vial}}{\text{milligrams wet tissue per vial}}$$

3.3.2(b). Results.

Whilst there was some variability in thymidine uptake between doses, no clear dose dependent effect of cortisol was apparent *in vitro* (Figure 3.3). Indeed, analysis of variance revealed no statistically significant effect of cortisol dose on the rate of DNA synthesis (F ratio of 1.77, not significant at the 5% level). In other words, there was no *in vitro* effect of four hours of treatment with cortisol at these dose levels in skin removed from an adrenalectomised animal.

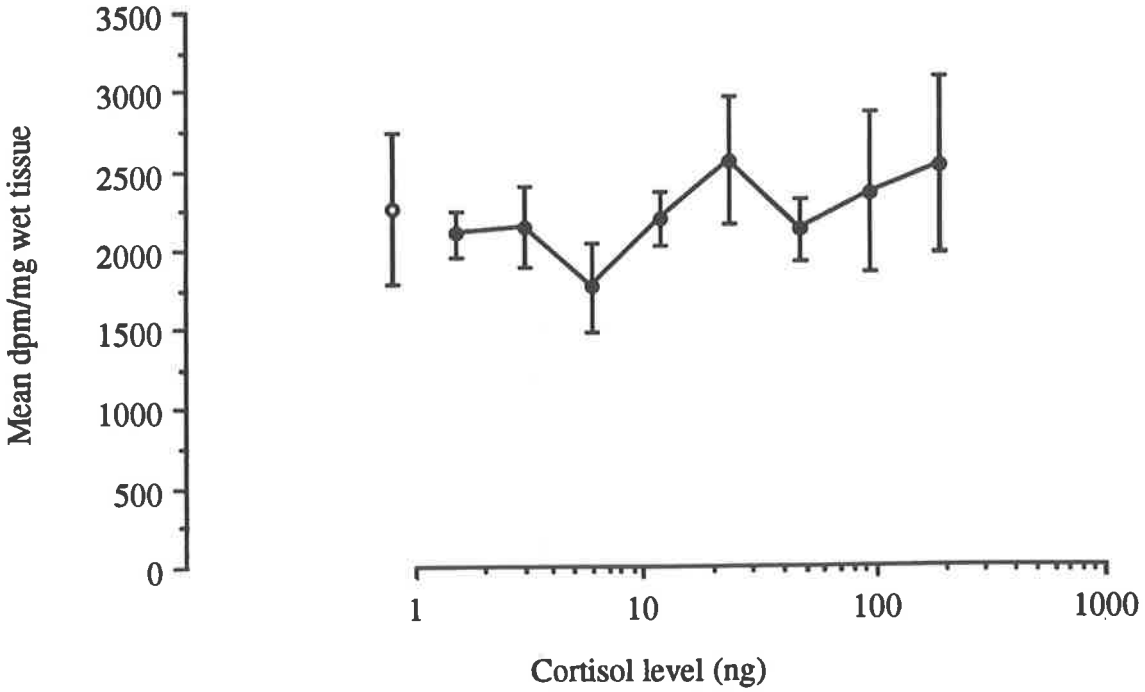


Figure 3.3. The effect of various levels of cortisol added to 3.2ml of culture medium on the *in vitro* incorporation of [^{3}H]thymidine into strips of sheep skin (disintegrations per minute per milligram of fresh tissue).

Control samples (∘) had no cortisol added (i.e. 0ng). Treated samples (•), and associated s.e.m. indicated by error bars.

3.3.3. Discussion: Experiments 3a and 3b.

Clearly, the rate of cell division and DNA synthesis were unresponsive to local cortisol treatment within two to six hours. The following experiment was designed to examine whether cortisol exerts any controlling influence on cell division when it is present in the circulatory system.

The concern of Experiment 3a was purely to determine the effect of locally-administered cortisol on a follicle population. The skin biopsies studied were removed from individual animals, thus the response of a large number of individual follicles ($n > 200$) of identical genetic origin was examined.

3.4. Investigation of the effects of changes in systemic cortisol levels.

Since locally-administered cortisol had no short term effect on the processes involved in wool growth from the follicle, the following experiments were undertaken to test the hypothesis that an increase in the level of systemic cortisol would reduce the rate of mitosis in the wool follicle. That is, there may be some factor which relates to whole body stimulation with cortisol that can influence cell division in the short term.

3.4.1. Experiment 3c.

The aim of the following experiment was to determine whether acute stimulation of the adrenal gland in entire animals would result in a reduction in the cell division rate in wool follicles.

3.4.1(a). Experimental Procedure.

Four entire animals (Ent1 to Ent4) were housed and fed as above. Catheters were inserted into the jugular vein one day prior to the beginning of the following skin and blood sampling regime. Heparinised blood samples (10ml) were collected from each animal at one hour intervals from 1300 hours to 1800 hours. At 1600 hours, intradermal injections of colchicine were made into sites overlying the tenth rib, marked on the right midside of each animal and skin biopsies were removed at 1800 hours and treated as per the method of Hynd *et al.* (1986).

Blood sampling and skin sampling (from a similar site on the left midside) was repeated the following day at the same times, and an injection of ACTH was given to each animal at 1400 hours. Additional blood samples were collected at 1330, 1420 and 1440, according to the regime fully described above in Section 3.2.6(a). This provided skin samples taken at the same time of day from the same animals, under conditions of low and high cortisol of an endogenous origin.

3.4.1(b). Results.

As previously shown above (Figure 3.2), plasma cortisol levels began to rise immediately after the injection of ACTH, and continued to rise over the entire four hour period. The plasma cortisol level on day one was 2.3 ± 8.3 ng/ml, and that on the following day was 76.3 ± 54.6 ng/ml, based on the average of the plasma samples collected at 1300, 1400, 1500, 1600, 1700 and 1800 hours each day. A simple average has been calculated, because it describes a gross difference (2.3 vs 76.3) in plasma cortisol between the two groups of plasma samples collected during the two days. There was a great deal of variability in the cortisol levels recorded in the samples, which was caused by two samples with high cortisol levels (9.4 and 40.1 ng/ml) in two animals on the first day, and steadily increasing levels on the second as illustrated in Figure 3.2.

The following table shows the average cell division rate for each animal on the two consecutive days. Despite the vast differences in plasma cortisol for all of the animals, there was no significant difference in cell division rate between the two days for individual animals when examined using the Kolmogorov-Smirnov test. Similarly, there was no significant difference between the average of all animals between the two days, using the students t-test.

Table 3.3. The effect of very low (Day 1), and very high (Day 2) levels of endogenously released cortisol on cell division rate.

	Day 1	Day 2
Ent1	4.30[a]	4.01[a]
Ent2	4.73[a]	4.71[a]
Ent3	4.51[a]	4.79[a]
Ent4	4.18[a]	4.28[a]
Average	**4.43 ± 0.24[b]**	**4.45 ± 0.37[b]**

[a] Not significant according to the Kolmogorov-Smirnov test (within sheep).

[b] Not significant according to the t-test.

3.4.2. Experiment 3d.

The following experiment was designed to determine whether mitosis in the wool follicle can be influenced by an intravenous dose of cortisol administered as a bolus.

3.4.2(a). Experimental Procedure.

Eight adrenalectomised animals (Adx21 to Adx28) were housed in individual pens, and fed a ration as above. DOCA was administered, but cortisol was omitted each day for six days. On the sixth day, jugular catheters were inserted into each animal. On the seventh day, heparinised blood samples (10 ml) were collected at 1300, 1400, 1500, 1600, 1700 and 1800 hours. At 1600 hours, intradermal colchicine injections were made into a site lying over the tenth rib on the right midsideof each animal and skin biopsies were removed at 1800 hours and treated as per the method of Hynd *et al.* (1986).

The following day, hydrocortisone sodium succinate, equivalent to 200 mg of hydrocortisone (Solu-Cortef, Upjohn Pty Ltd, 55-73 Kirby Street, Rydalmere N.S.W. 2116) was injected as a bolus (4 ml) via the jugular catheter of four sheep (Adx21 to Adx24) and an equivalent amount of sodium succinate alone into the remaining four sheep (Adx25 to Adx28). Injections were commenced at 1245 and were completed by 1255 hours. Blood and skin sampling procedures were repeated as before, with the exception that an equivalent site on the left midside was used for the colchicine injection and biopsy.

3.4.2(b). Results.

Despite the large differences in measured cortisol, there was no significant difference between the average rate of cell division for any of the treatments. The plasma cortisol levels attained by the bolus injection in the treated group are illustrated in Figure 3.4.

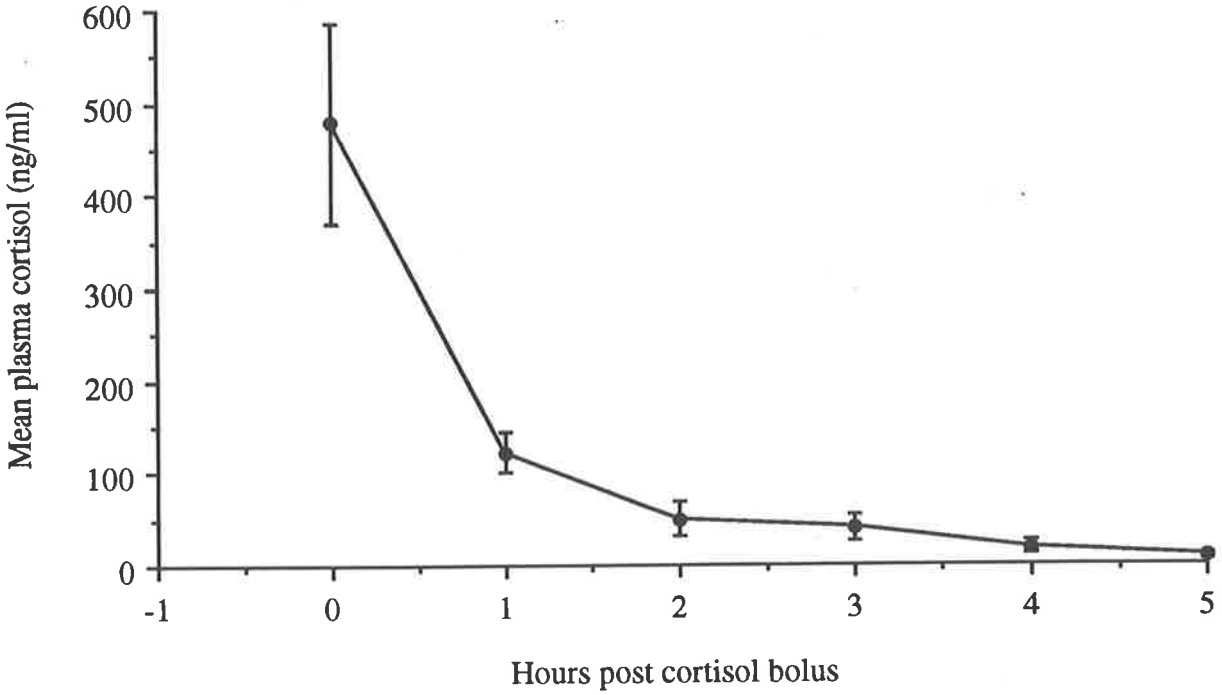


Figure 3.4. The decline of plasma cortisol with time after an intravenous bolus injection of cortisol sodium succinate in adrenalectomised animals. Group mean (•), and associated s.e.m. indicated by error bars.

On the first day of blood sampling (day 7), plasma cortisol levels were negligible in all eight animals. One animal from each group showed some plasma cortisol, a high of 7 ng/ml for the control group (Adx25), and 2 ng/ml for the treatment group (Adx23). The following day, the control group similarly showed negligible cortisol. Animal Adx25 exhibited some cortisol, with no evidence in the other animals. The treated group on the other hand, showed very high levels (478 ± 216 ng/ml) of cortisol in the plasma at 1300 hours, which rapidly diminished over the following five samples to reach an average of 8 ng/ml (± 7 ng/ml) at 1800 hours when the skin samples were removed.

Once again, there was evidence of cortisol secretion in one animal in the untreated group in two of the six samples taken on day 7, and three of the six samples on day 8. This suggests that there was also surviving adrenal tissue in this animal, and although this was not located during post experimental autopsy, this animal was not challenged with ACTH and the ability of the source to respond to stimulation remains unknown. The highest single recorded value in this animal was 9 ng/ml, which was regarded as negligible given the difference between this and the levels attained in the treated group.

The following table shows the cell division rate for each of the animals on both of the days, and the mean for each group. Analysis of variance revealed that over all the animals, there was a significant reduction in cell division rate between day 6 and day 7. However there was no difference between the two groups on either day, which shows that the cortisol treatment had no effect on cell division despite the large difference in plasma cortisol levels between the two groups.

Table 3.4. The mean rate of cell division in adrenalectomised animals during a control period (Day 6) of low plasma cortisol, and low (untreated group) or high (treated group) plasma cortisol (Day 7).

		Day six	Day seven
		Low cortisol	High cortisol
Treated Group	Adx21	4.27	3.20
	Adx22	5.47	4.65
	Adx23	3.56	4.14
	Adx24	6.62	4.88
	Average	**4.98 ± 1.35**	**4.22 ± 0.74**
		Low cortisol	Low cortisol
Untreated Group	Adx25	2.76	2.30
	Adx26	4.46	3.58
	Adx27	3.27	3.60
	Adx28	5.80	5.54
	Average	**4.07 ± 1.35**	**3.75 ± 1.34**

3.4.3. Discussion: Experiments 3c and 3d.

Experiments 3c and 3d show that a short term change in systemic cortisol does not influence the rate of cell division. Neither four hours of increasing endogenous hormone levels after ACTH stimulation, nor five hours of falling levels after an exogenous bolus produced any change in cell division rate.

Cortisol was evident in the plasma of the unstimulated entire animals in Experiment 3c and in view of the episodic nature of cortisol release, it is possible that hourly sampling may have overlooked isolated peaks in hormone concentration. However, as has been stressed previously, restraint and sampling itself can lead to stimulation of the adrenal cortex; the sampling regime was a compromise between accurate assessment of cortisol level and provoking endogenous release on day one. With due respect to this argument, the magnitude of the differences in hormone levels produced by the ACTH stimulation was considerable (2.3 vs 76.3 ng/ml, for the control and treatment periods respectively) and was sustained throughout the entire four hour period, without any evidence of an accompanying change in cell division rate measured over the last two hours. When coupled with the results of Experiment 3d it was concluded that no more useful data would be obtained by implementing a blood sampling regime of increased frequency.

The decrease in mitotic rate observed on day two with respect to day one of Experiment 3c may be an effect of the sodium succinate, the time elapsed between withdrawal of cortisol and the experimental recordings or an environmental difference between the two days. Based on the change observed in the control animals, a covariate analysis was performed in this case.

Experiment 3c and 3d reinforce the observations of Experiment 3a and 3b. Once again, conditions of dramatically increased plasma cortisol were produced by the techniques yet there was no concurrent change in cell division rate. In conclusion, changes in plasma cortisol level of two to five hours in duration, either in the blood stream or locally in the skin, exert no influence on mitotic rate. Since the rate of wool growth is correlated with the number of mitoses arrested per bulb (Schinckel, 1961, 1962; Hynd *et al.*, 1986) by inference we can say that wool growth would similarly be unaffected. The literature shows that there is a measurable effect of cortisol on wool growth after four days (reviewed by Wallace, 1979a), while the evidence presented here shows there is no change in the short term. It was therefore of interest to determine exactly what time period is required before a change in glucocorticoid status exerts an influence on cell division rate.

3.5. The minimum time period for cortisol to influence cell division rate.

3.5.1. Experiment 3e.

The following experiment was designed in an effort to determine a dose level of intravenously-infused cortisol, which would produce a level of plasma cortisol likely to affect wool growth rate. Skin biopsies were taken in conjunction with this experiment to indicate whether a change in cell division rate becomes apparent after three days of various increases in cortisol level.

3.5.1(a). Experimental Procedure.

Bilateral jugular catheters were inserted into eight adrenalectomised wethers (Adx29 to Adx36). The following day the animals were placed in metabolism crates, and after one week, cortisol therapy was omitted. Restricted feeding was imposed (750g of lucerne chaff per day) but unlimited water and salt (NaCl) were provided.

During the ensuing experimental period, various levels of cortisol dissolved in physiological saline were administered via the right jugular catheter. For five consecutive three day periods the daily infusion of saline contained 0, 5, 10, 20, and 40 mg of cortisol. Solubility of the steroid was achieved by initial dissolution in propylene glycol (Propane-1,2-diol, G.P.R. grade, B.D.H. Chemicals, Port Fairy, Vic. 3284) to produce a concentrated stock solution. The stock solution was passed through 0.22 μm sterile filters to achieve sterility (Millipore Products Division, Bedford, Massachusetts, 01730, USA) and serial dilutions of the stock prepared so that 5 ml of each dilution was mixed with the daily saline infusion.

The infusate was changed at 0900 hours every day and the animals were fed at this time. Blood samples (10 ml) were collected into heparinised tubes from the left jugular catheter every fifteen minutes beginning at 1200 hours and finishing at 1245 hours every day. On the first and third day of each three day infusion period, the intradermal colchicine method was used to obtain skin samples at 1500 hours (injections at 1300 hours) from either side of the animals according to the pattern illustrated in Figure 3.5.

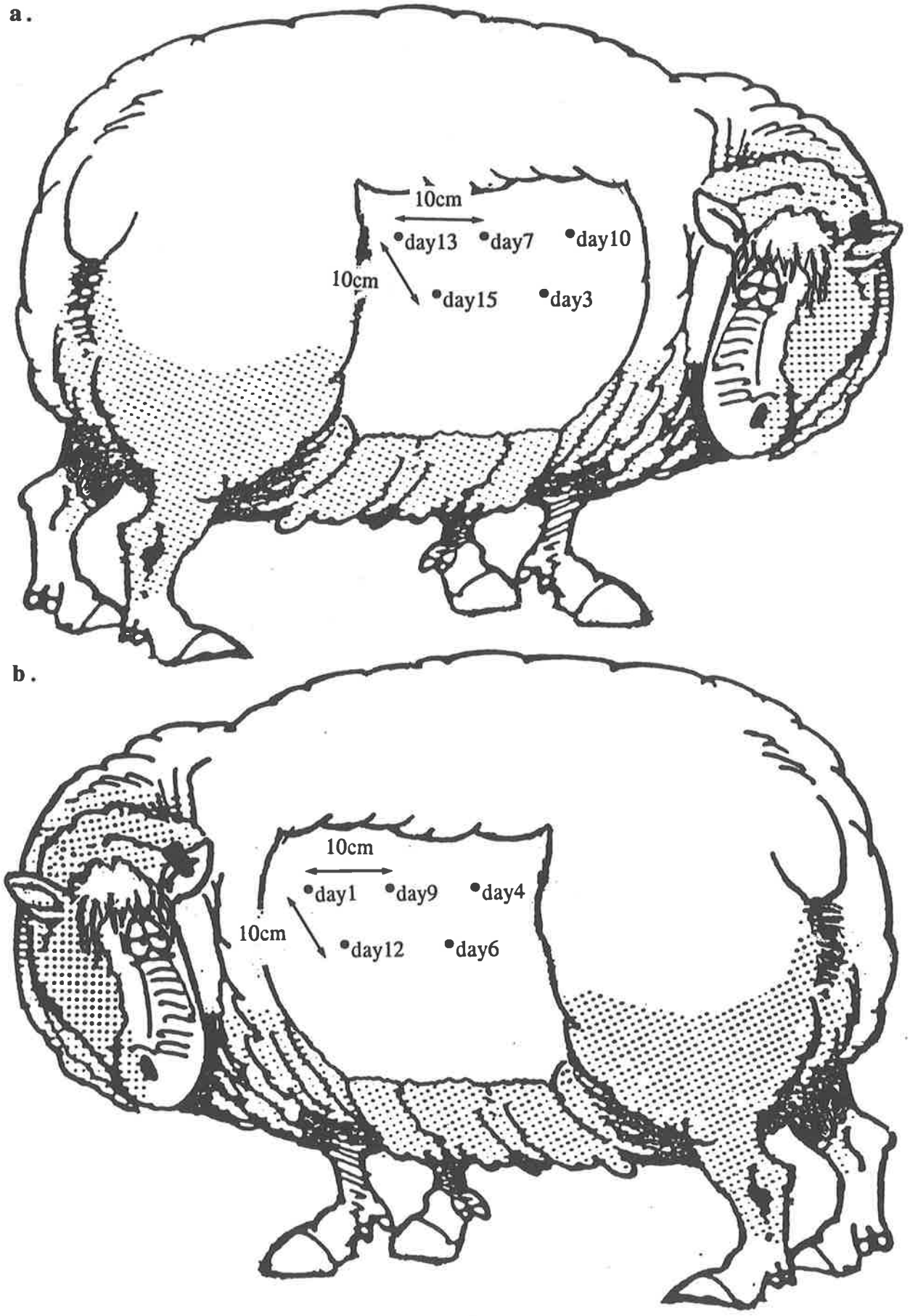


Figure 3.5. The sites and day of sampling of biopsies collected from adrenalectomised wethers (a) on the right midside and (b) on the left midside, in Experiment 3e. Adapted from Ball (1979).

Assay of plasma cortisol.

Plasma cortisol levels were determined using Amersham RIA kits with the following modifications applied to the instructions included in the kit.

The volume of unknown plasma sample added was 20µl rather than 50µl as recommended in the kit. The amount of both [^{125}I]-cortisol and antibody suspension was reduced to 50µl from the 200µl recommended. Additional zero standard (Amersham Laboratories, White Lion Road, Amersham, Buckinghamshire, England, HP79LL) was used to prepare dilutions of the lowest cortisol standard, and to dilute all standards provided with the kit. The final set of standards contained cortisol at levels of 0, 0.6, 1.2, 1.8, 2.4, 3, 12.5, 33, 84 and 175 ng/ml. Using this procedure, the between-assay coefficient of variance was 12%, and the within-assay coefficient of variance was 8%.

(The modifications to the procedure were provided by courtesy of Elke Henning and Tim Kuchel of the Flinders University of South Australia, who kindly carried out the assays for this experiment.)

3.5.1(b). Results.

Unfortunately two of the sheep (Adx33 and Adx36) used in this experiment died and a further three (Adx29, Adx30 and Adx35) had to be removed from the experiment within the first three days to save their lives. This could have been due to the prevailing weather conditions. Although the animals were kept indoors, they were not enclosed in a controlled environment room and environmental temperatures were very high during the first few days of the experiment.

Three sheep (Adx31, Adx32 and Adx34) survived the entire treatment period under the conditions imposed. A definite increase in plasma cortisol levels was produced by the infusion at dose rates above 5 mg of cortisol per day. Plasma cortisol levels in excess of 20 ng/ml were brought about by the highest dose. A high level of variability was observed between plasma

cortisol levels in samples collected from the left jugular during each one hour period. This was evident at all levels of cortisol administration above 5 mg per day, despite continuous infusion of the corticosteroid into the left jugular throughout the same period.

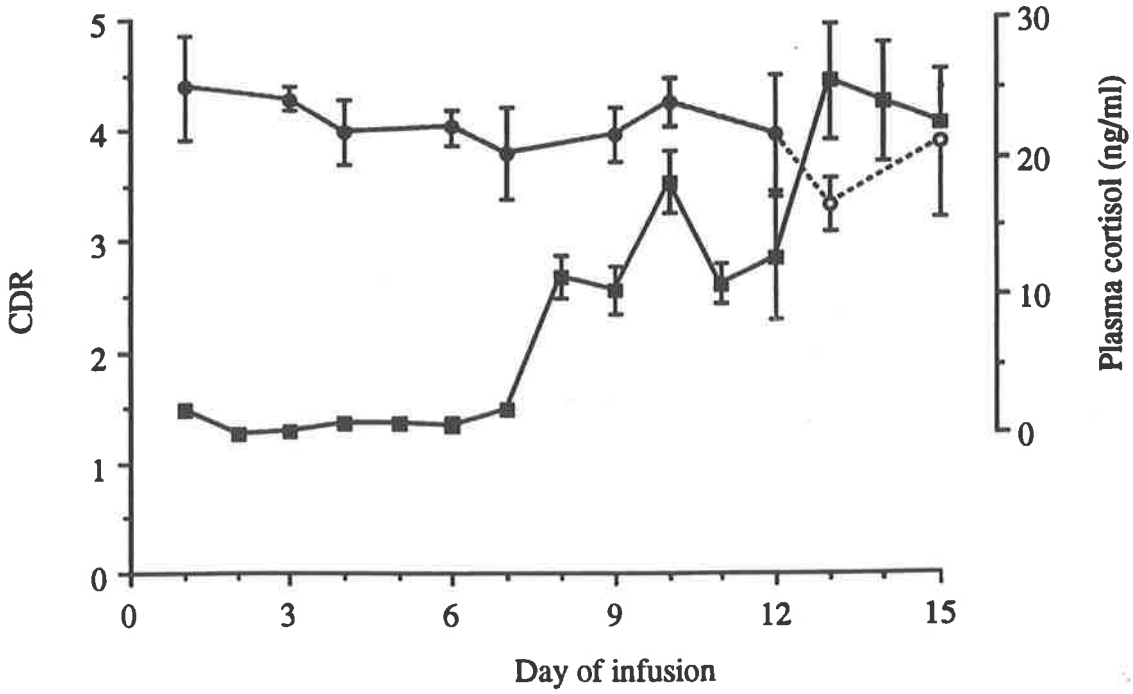


Figure 3.6. Plasma cortisol levels (■) produced by increasing levels (0, 5, 10, 20, 40 mg/day) of infused cortisol for periods of 3 days, and the accompanying changes in cell division rate (●). Beyond day 12 only two animals were recorded for cell division rate, which is indicated by (○).

As shown in Figure 3.6, there was no marked effect of an increase in plasma cortisol levels on follicle mitotic activity. More importantly, a period of six hours elapsed between the transition from one cortisol infusion rate to the next (at 0900 hours) and the removal of skin biopsy samples (at 1500 hours) on the first day of all infusion levels. The rate of cell division was not significantly depressed in any of these samples, when compared to that in the sample taken one day previously.

Cell division rate measurements were not available for one animal (Adx34) on day 13, as the sample was spoilt during processing. Much of the variation in measured plasma cortisol rates was caused by this same animal, in which differences between samples collected beyond day 10 ranged between 0 and 30 ng/ml.

3.5.1(c). Discussion.

The first six days of the infusion were a period of very low (undetectable) plasma cortisol levels in the survivors (Adx31, Adx32 and Adx34) of Experiment 3e. (N.B. "Undetectable" plasma cortisol was a level of less than 0.6 ng/ml. T. Kuchel pers. comm.). Despite the difficulties experienced during Experiment 3e, it was possible to increase plasma cortisol from these low levels up to levels well in excess of 10 to 12 ng/ml in three sheep. Thus the goal of the experimental manipulations was achieved.

The highest of the plasma cortisol levels were within the range shown by Chapman and Bassett (1970) to reduce wool growth rates after four days. In the present experiment the very highest levels were maintained for a maximum of three consecutive days with little effect on mitotic rate. This suggests that the cell division component of wool growth was unchanged by elevated plasma cortisol in sheep under restricted feeding conditions, over periods of less than four days. Levels of 10 ng/ml and greater were maintained for a period of six days in total, however this failed to significantly depress cell division. The highest infusion rate of the present experiment formed the basis for the lowest level administered in Experiment 3f (below).

In support of the results of Experiments 3c and 3d, the rate of mitosis in the wool follicle was not affected by a change in the rate of infusion of cortisol from one day to the next. As noted in the results section above, there was a period of six hours between the time at which the cortisol infusion was changed and the time at which skin biopsies were collected. Blood samples were collected three hours after changing the infusion, and in general indicated an increase in plasma cortisol from one level to the next. There was no change in cell division associated with the increase in cortisol level, thus we can add that on no occasion did an increase in plasma cortisol reduce the rate of cell division within six hours.

3.5.2. Experiment 3f.

The aim of the following experiment was to determine the number of days of elevated plasma cortisol necessary to reduce the rate of cell division in wool follicle.

3.5.2(a). Experimental Procedure.

Fifteen adrenalectomised animals (Adx1 to Adx16) were placed in a controlled environment room, (24 ± 2°C, 12 hours of light commencing 0700 hours). Bilateral jugular catheters were inserted two days prior to commencement of the experimental period. The animals were maintained with DOCA injections as described previously. The animals were fed 1 kg of a pelleted ration, plus 100g of chopped lucerne hay, at 0900 hours.

On the initial day of the experiment, a solution of physiological saline containing 40ml of propylene glycol per litre, was infused via the left jugular catheter at a rate of 10 ml per hour. Infusion was commenced at 1300 hours on day 1 and the infusion rate was controlled by infusion pumps (Gilson, France). From the right jugular catheter, six heparinised blood samples (10 ml) were collected each day at one hour intervals commencing at 1300 hours. At 1600 hours each day, colchicine injections (50μg) were administered and skin biopsies removed at 1800 hours from each animal according to the pattern illustrated in Figure 3.7.

For days one to four, and six to twelve the level of plasma cortisol was determined in pooled plasma samples consisting of equal proportions of the six samples. All six samples were assayed individually for plasma cortisol collected on day five, to ensure that the level of cortisol was increased prior to the collection of the skin samples on this day.

Commencing on day 5 at 1300 hours, cortisol was added to the infusate administered to ten of the animals. Five animals received cortisol at a dose based upon the highest rate from Experiment 3e, i.e. 40 mg/day. Five animals received almost double that of the first group. Control animals received only saline with propylene glycol solution throughout the entire period. On the basis of body weight of the individual animals and residual solution at the end of each infusion period, actual infusion rates were calculated, and averaged 0.87 and 1.82 $mg.kg^{-1}.day^{-1}$ for the low and high infusion rates respectively.

At 1300 hours on day 9 all animals were returned to the control infusate. The infusion was terminated at 1800 hours on day 12 after the last of the skin biopsies were collected.

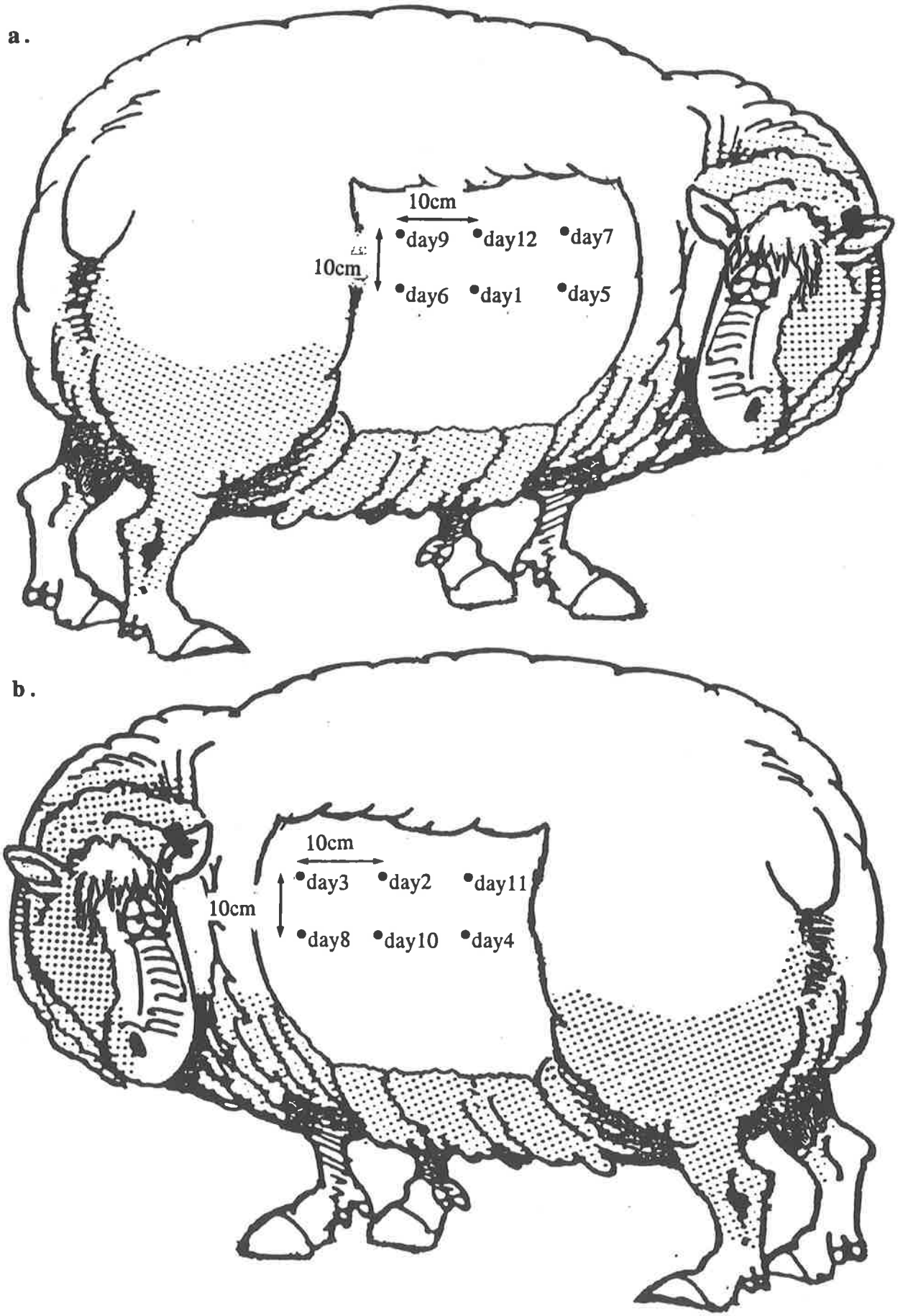


Figure 3.7. The sites and day of sampling of biopsies collected from adrenalectomised wethers (a) on the right midside and (b) on the left midside, in Experiment 3f. Adapted from Ball (1979).

3.5.2(b). Results.

Plasma cortisol levels were manipulated smoothly by the procedure. Animal Adx15 was excluded from the results based on the knowledge that it had a small piece of functional adrenal tissue (from Section 3.2.6(b). above). Although there was a small amount of cortisol recorded in other members of the control group, none of these were responsive to ACTH (Section 3.2.6(b)) and the highest recordings were 2.4 ng/ml for the pooled estimates and 7.1 ng/ml for individual samples collected on day 5, both in animal Adx12. For the rest of the control group, the highest recording was 2.4 ng/ml for a pooled estimate from animal Adx10 on day 9, and 10.5 ng/ml for a single sample collected on day 5 from animal Adx1. The highest of these recordings, (10.5 ng/ml) reached less than one third of the lowest level recorded in the group infused with the low dose.

The low dose infusion caused the average plasma cortisol level to increase rapidly over the first five hours of the infusion, as shown in Figure 3.8. Over the following three days of infusion the average plasma cortisol level was 65.4 (± 39.5) ng/ml, illustrated in Figure 3.9. Variability of the level of cortisol was high within sheep in this group, the lowest pooled estimate recorded from an individual animal was 25.2 ng/ml and the highest 139.1 ng/ml. From Figure 3.4 the disappearance of plasma cortisol was relatively rapid after a large single bolus dose; the lowered plasma levels recorded in this group on day 9 reflect a similarly rapid decline. These levels were determined as a pooled estimate rather than individual recordings as on day 5.

Finally, the high dose rate infusion resulted in extremely high plasma cortisol levels within the first five hours of the commencement of infusion. The highest recordings were 302.6 ng/ml in individual samples collected on day five and 439.4 ng/ml in pooled samples. The average level calculated from pooled estimates for days 6, 7 and 8 was 158.2 (±128) ng/ml. Once again, variability within sheep was high, but the average was slightly more than double that of the low dose group, and much higher than that observed in the control animals.

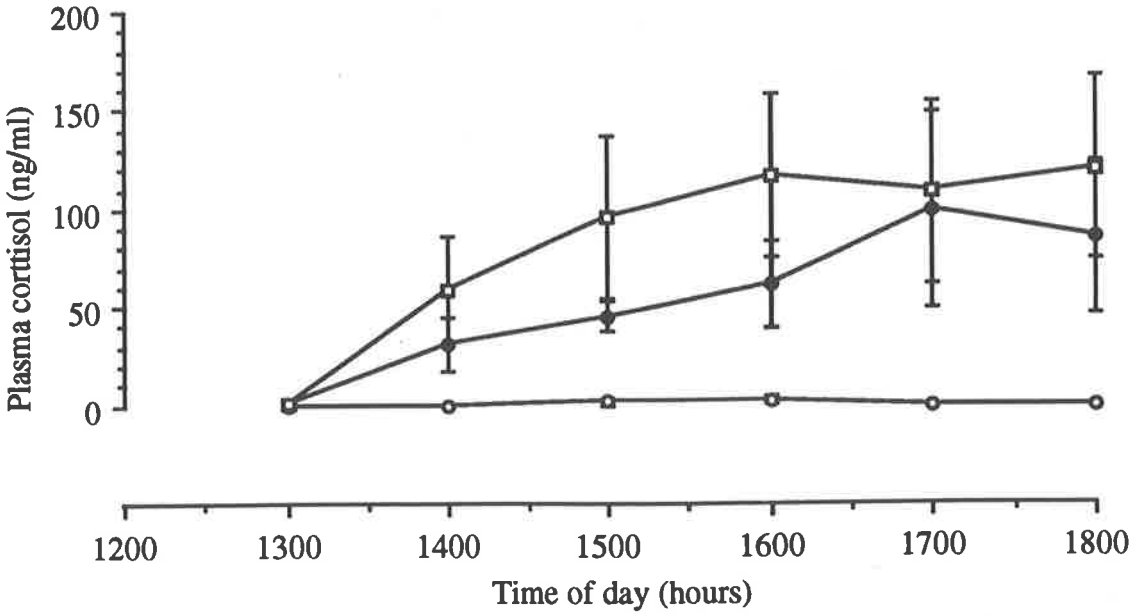


Figure 3.8. Group mean plasma cortisol levels produced by infusion of 0 mg.kg^{-1}.day^{-1} (o), 0.87 mg.kg^{-1}.day^{-1} (●) or 1.82 mg.kg^{-1}.day^{-1} (▫) during the first five hours of infusion on day 5.

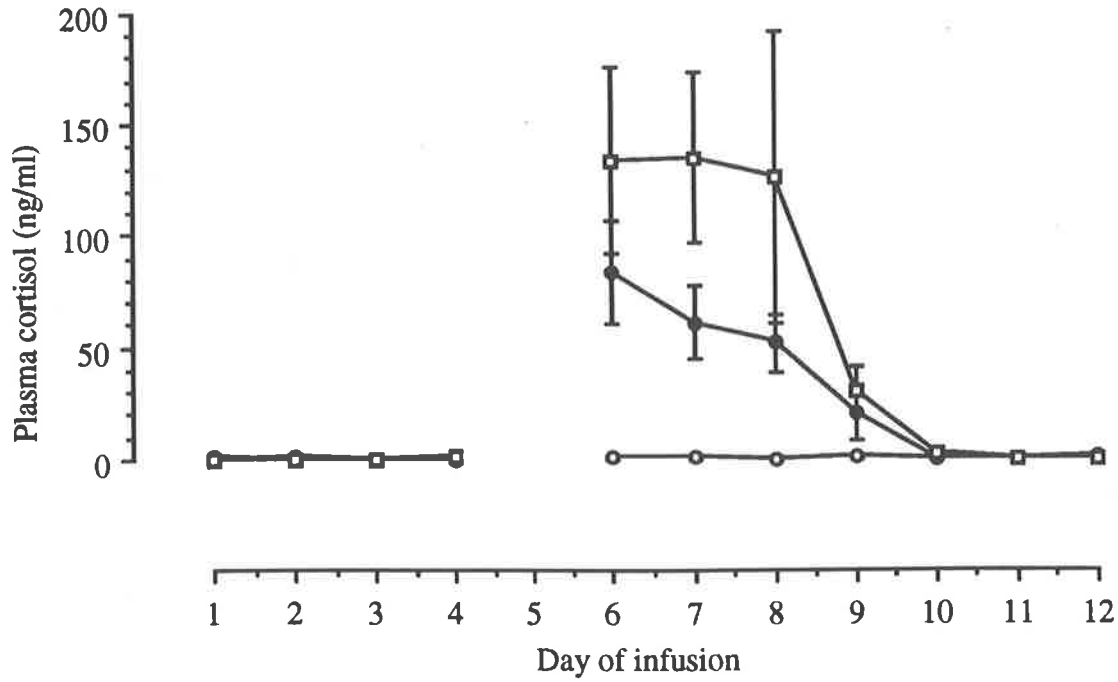


Figure 3.9. Group mean plasma cortisol levels produced by infusion of 0 mg.kg^{-1}.day^{-1} (o), 0.87 mg.kg^{-1}.day^{-1} (●) or 1.82 mg.kg^{-1}.day^{-1} (▫) during day 5 to day 9.

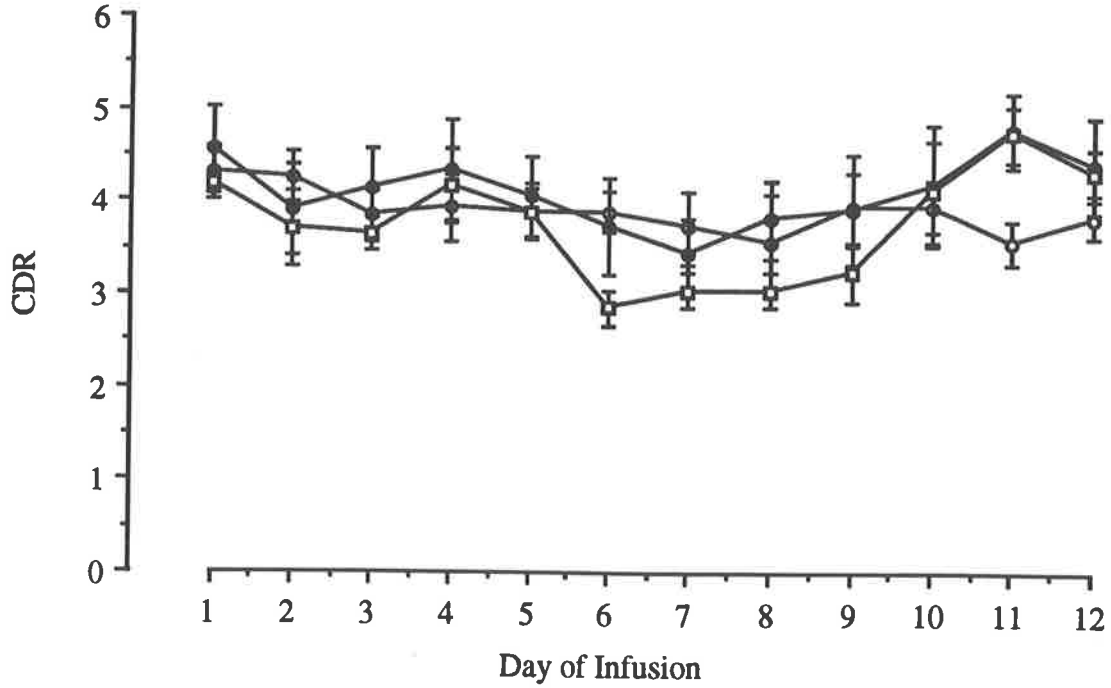


Figure 3.10. Cell division rate prior to (day 1 to 4), during (day 5 to 8), and after (day 9 to 12) infusion of cortisol into adrenalectomised animals at three different levels (0 (∘), 0.87 (●) and 1.82 (▫) mg.kg^{-1}.day^{-1}).

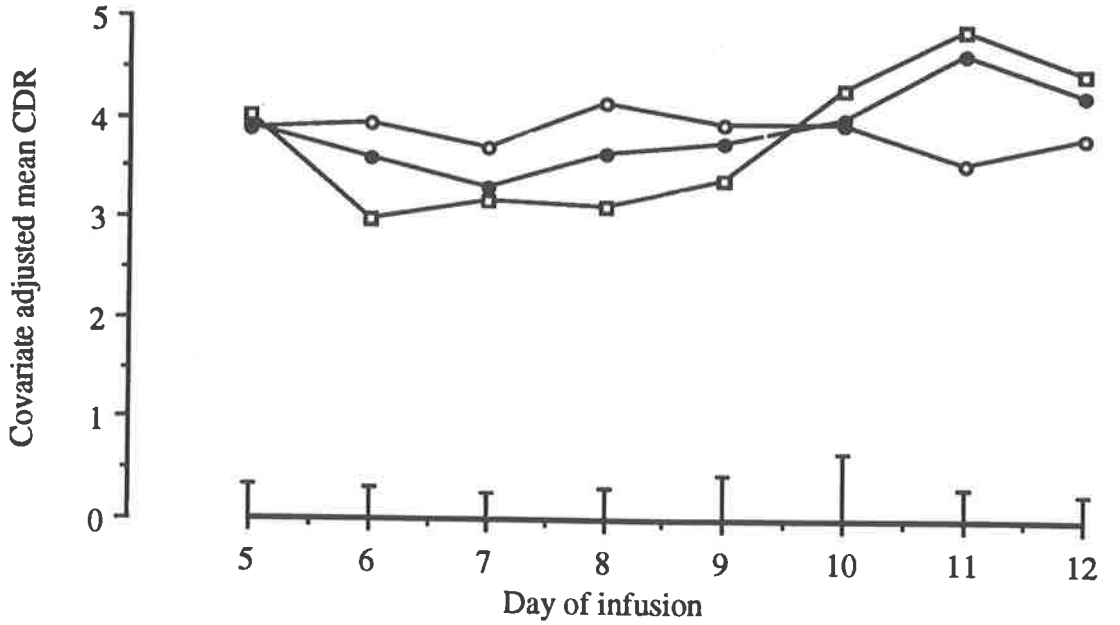


Figure 3.11. Mean cell division rate during cortisol infusion (day 5 to 8) and post-infusion (day 9 to 12) periods, adjusted using pre-infusion (day 1 to 4) cell division rate as a covariate. Cortisol was infused at three different levels (0 (∘), 0.87 (●) and 1.82 (▫) mg.kg^{-1}.day^{-1}). Error bars on the x-axis represent the standard error of the differences between means.

Consistent rates of cell division were recorded in the biopsies collected from all of the control animals. One animal of the control group, Adx15, was excluded from the analysis and the data from this animal were excluded from all of the Figures 3.8 to 3.11. It is of some importance to note that the cell division rate recorded in this animal was comparable to other animals of the control group and showed no change throughout the 12 day period. The sole reason for excluding this animal was that it had surviving adrenal tissue which was responsive to ACTH, and was therefore unlike all of the other animals. Although animal Adx12 showed sound evidence for the secretion of cortisol, the level of the hormone in the plasma was very low and unresponsive to ACTH (see Section 3.2.6(a). above). Animal Adx12 was included in the analysis, and in all of the Figures 3.8 to 3.11.

Between sheep, the rate of cell division was very variable, yet within sheep it was remarkably repeatable. Samples collected from the animals in the low dose infusion group showed the greatest between-sheep variance. It was for this reason that an analysis of covariance was performed on the data, and the group means for cell division rate, adjusted using days one to four as the covariate period are displayed in Figure 3.11. All animals exhibited a slight reduction in cell division rate between days one and two, which may reflect an adjustment to the infusate.

There was a decline in cell division rate between day five and six in both cortisol-treated groups, which continued for the following three days of cortisol infusion. The reduction was significant on days six, seven and eight for the group which received the highest rate of cortisol infusion, and became non-significant on day 9, with subsequent recovery so that by day 10, mitotic rate had increased to a level similar to that of the control period recordings. Although the reduction was not significant for the group which received 0.87 $mg.kg^{-1}.day^{-1}$, cell division rate ranged at an intermediate level in this group which suggests a dose-dependent effect. By day 11, a significant increase in cell division rate, above that recorded in control animals, was evident in both cortisol-infused groups. This was also evident on day 12, although slightly smaller in magnitude. Indeed, rates of cell division recorded in all the animals which received cortisol, were higher after cortisol infusion ceased than their respective cell division rates recorded during days one to four of the infusion period.

3.5.2(c). Discussion.

The most important points to note are that in none of the treatment groups was the rate of cell division significantly changed within the first five hours of a change in the level of cortisol infused into the jugular. The average cell division rate on day 5 was no lower than it had been for the four preceding days, and it failed to increase on day 9 with respect to the preceding two days.

Once again, the rate of cell division had not changed after five hours of increasing levels of plasma cortisol, which shows that rapid changes in plasma cortisol have little short-term effect on the rate of cell division. The greatest reduction in cell division rate was seen in skin samples collected from animals which received the highest level of infusion, with an intermediate level of cell division in the group which received the low level of infusion, suggesting dose dependence of the effect. Although the differences in mitotic rate were not significant on day 9, an effect of a slight recovery after cortisol infusion ceased, but more importantly a reflection of the steady decline in cell division in the group which received the control infusion.

An important point to note is that on day 11, fifty-four hours after all eight cortisol infused animals had been returned to the control infusion, cell division rate increased to a level significantly greater than that recorded from the animals during the pre-cortisol infusion, and remained so on day 12. Recovery after treatment ceased, to levels of wool growth higher than pre-treatment was apparent for growth hormone reviewed in Chapter One (Ferguson, 1951, 1954; Ferguson *et al.*, 1965; Wheatley *et al.*, 1966; Wallace, 1979a). In all cases, the recovery period was observed at times longer than fifty-four hours after cessation of treatment, however the techniques used to the obtain the measurements do not possess the sensitivity of the mitotic technique, as considerably longer periods are required.

Since cell division rate in the group which received only 0.87 $mg.kg^{-1}.day^{-1}$ was not significantly lower than the control, the lack of a response in cell division rate during Experiment 3e could thus be due to much lower levels of plasma cortisol produced by the highest infusion rate imposed in that experiment.

During the present experiment, a constant infusion of high, low and zero doses of cortisol resulted in high, intermediate and very low circulating levels of the hormone, which were highly variable rather than consistent. Rather than detracting from the experiment, the variability of plasma cortisol was considered to be of some value, as studies of circulating levels in entire animals show a great deal of variability and pulsatile release patterns (McNatty *et al.*, 1972; Holley *et al.*, 1975; Fulkerson and Tang, 1979). Normal fluctuations in the level of plasma cortisol in sheep may indeed be necessary to maintain wool growth, as there was a steady decline in cell division in the control infusion group and an increase in cell division when cortisol infusion ceased in the treated groups.

3.6. General Discussion on the short term effects of cortisol.

As shown in Chapter One, there is a comprehensive body of literature which shows that cortisol can strongly influence the rate of wool growth. The results of Experiment 3a show that over the range of doses used, intradermal cortisol had little effect on cell division *in vivo*. There was no consistent significant effect either between the doses used or between the times of exposure to exogenous cortisol.

Similarly, from the results of Experiment 3b it was apparent that the rate of DNA synthesis *in vitro* was not influenced by the presence of cortisol in the medium. That is, there was no significant difference in the rate of incorporation of [^{3}H]thymidine between the various dose levels.

All treatments in the literature were of much longer duration than the present experiment and, as Wallace (1979a) pointed out, the minimum time required for an effect of cortisol to become evident has never been determined. This thesis has resolved this question, at least in terms of cell division. During the experiments reported here, periods as short as two hours were studied in the *in vivo* experiments and four and a half hours in the *in vitro* experiments. Unpublished evidence of K.A. Ward (cited by Wallace, 1979a) apparently produced a similar result, although no detail or evidence was reported. Thus, on a local basis cortisol influences the functioning of wool follicles very little in the short term. This is interesting, since previous

authors have shown that cortisol has profound effects on the rate of wool growth (Lindner and Ferguson, 1956; Ferguson *et al.*, 1965; Chapman and Bassett,1970). Obviously elevated levels of cortisol must be maintained for long periods to suppress the cell division component of wool growth.

In conclusion, all of the experiments reported in this chapter suggest that fluctuations in cortisol levels throughout the day would produce neither the rhythmic fluctuations in cell division rate observed by Scobie and Hynd (1987) nor the erratic fluctuations observed in the experiment conducted in Chapter Two. The following chapter examines the possibility that the observed changes in cell division rate are a result/artifact produced by the levels of catecholamines.

The relationship between circadian fluctuations in depilation force measurements and cortisol levels suggested by Foldes *et al.* (1985) is not contradicted by the evidence presented here. It is possible that cortisol may influence wool growth at the level of fibre keratin formation rather than cell division. The strength of attachment of fibre to the skin could vary with keratin synthetic rates and independently of cell division rate.

Chapter Four.

The short term effects of catecholamines on cell division.

"From another direction he felt the sensation of being a sheep startled by a flying saucer, but it was virtually indistinguishable from the feeling of being a sheep startled by anything else it ever encountered, for they were creatures who learned very little on their journey through life, and would be startled to see the sun rising in the morning, and astonished by all the green stuff in the fields.

He was surprised to find he could feel the sheep being startled by the sun that morning, and the morning before, and being startled by a clump of trees the day before that. He could go further and further back, but it got dull because all it consisted of was sheep being startled by things they'd been startled by the day before." (Adams, 1986).

4.1. Introduction.

Literature reviewed in Chapter One showed that the catecholamines affect wool growth, and the conflict as to whether these hormones act directly or indirectly to produce this effect was highlighted. Initial evidence suggested that vasoconstriction and reduced blood supply were the agents, and that catecholamine secretions from the adrenal gland were of little importance (Ferguson, 1949; Ferguson *et al.*, 1965). The results of Cunningham *et al.* (1979) showed that [^{35}S]cysteine uptake can be reduced by the presence of noradrenaline *in vivo*.

Using both *in vitro* and *in vivo* methods, the first of the following experiments was designed to determine whether the rates of DNA synthesis or cell division in the wool follicle, change under the influence of exogenously-introduced catecholamines. The later experiments of this chapter were undertaken to reveal whether the effect could be reproduced by release of catecholamines produced endogenously.

4.2. General Experimental Procedures.

4.2(a). Animals.

All animals used in the experiments described in this chapter were mature Merino wethers of fifty kilograms average weight. They were housed indoors and fed 800g of a pelleted ration, at 0900 hours each day. At least eight weeks under these conditions were allowed before any experiments were conducted. The individual animals were chosen from a large group, so that those with a less nervous disposition which readily adapted to shed conditions and handling were used for *in vivo* experiments. For the later experiments of this chapter, it was necessary to choose animals with a high level of face cover to ensure that skin samples could be taken from wool growing portions of the cheeks.

4.2(b). Chemicals.

The adrenaline (as the hydrochloride), noradrenaline (Arterenol hydrochloride) and the 6-OH dopamine (2,4,5-Trihydroxyphenethylamine) used in the following experiments were obtained from the Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178, USA.

4.3. Investigation of the effects of exogenous catecholamines.

4.3.1. Experiment 4a.

The following experiment was designed to determine the effect of intradermal doses of catecholamines on *in vivo* cell division in wool follicles.

4.3.1(a). Experimental Procedure.

Two sheep were used, and the injection sites were arranged according to the pattern outlined in Figure 4.1. At 1500 hours, two doses of noradrenaline (50 and 100μg) and two of adrenaline (50 and 100μg) were injected intradermally into each sheep, using 0.1 ml of saline as the vehicle for each dose. Saline (0.1 ml) was also injected at another site to act as the control sample. For sheep Cat1 colchicine (50 μg in 0.1ml of saline) was injected simultaneously into these sites and skin biopsies were taken two hours later and processed according to the method of Hynd *et al.* (1986). For sheep Cat2 a similar dose of colchicine was injected into all of the sites at 1700 hours, and skin samples removed at 1900 hours. This produced skin samples which had been treated with doses of noradrenaline and adrenaline for periods of two and four hours.

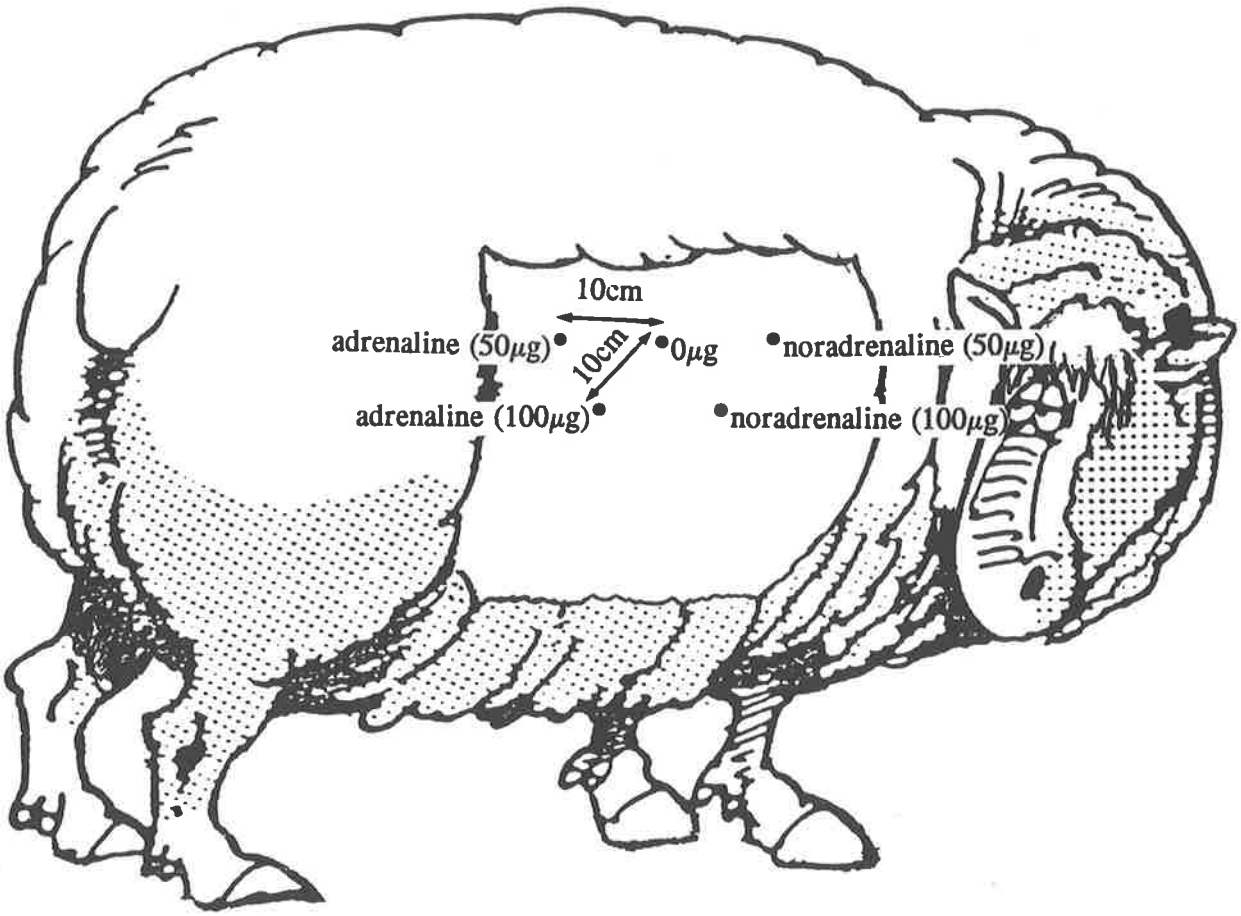


Figure 4.1. The site and dose of intradermal injections of catecholamines. Adapted from Ball (1979).

4.3.1(b). Results.

There was an obvious effect of the various intradermal doses of the catecholamines on cell division rate as shown in Table 4.1. Adrenaline significantly reduced cell division rate at the highest dose (100μg) after two and four hours when compared with the respective control samples. On the other hand noradrenaline significantly reduced cell division rates at both doses, two and four hours after intradermal injection. Thus cell division appeared to be more sensitive to noradrenaline than adrenaline.

Table 4.1. The influence of adrenaline and noradrenaline on mitotic rate after two and four hours.

	Sheep Cat1	Sheep Cat2
Duration of treatment.	2 hours	4 hours
Control	4.72	5.53
50μg noradrenaline	2.05[a]	3.58[b]
100μg noradrenaline	3.26[a]	2.14[b]
50μg adrenaline	4.42	3.78[b]
100μg adrenaline	2.96[a]	3.66[b]

[a, b] Significantly different to the control by the Kolmogorov-Smirnov test.

4.3.2. Experiment 4b.

The following experiment was designed to determine the effect of catecholamines on DNA synthesis in wool follicles *in vitro*.

4.3.2(a). Experimental Procedure.

Skin strips 1mm wide and approximately 20 cm in length were taken at 0900 hours from one sheep (Cat3) under local anaesthetic and treated as per the method of Ward and Harris (1976) with the following modifications. The strips were immediately immersed in warm culture medium (Medium 199 + Earles salts + Hepes 0.5% ascorbic acid) and placed in an incubator (37°C and 5% CO2). When a sufficient quantity of skin had been collected in this manner, it was cut into segments roughly 1.5 cm in length while immersed in the medium. Two such skin segments were placed at random in culture dishes containing a total of 3 mls of medium. To each culture dish, 5μCi of [^{3}H]thymidine (specific activity 9 Ci/mmol) (Amersham Laboratories, White Lion Road, Amersham, Buckinghamshire, England, HP79LL) in 0.1ml of saline was added.

Four levels of catecholamines (0.1 ml of saline containing 0, 0.625, 6.25, 62.5 mM solutions of adrenaline and of noradrenaline) were added to replicate culture dishes (n = 6) immediately prior to addition of the skin segments. The catecholamine solutions were prepared immediately prior to use and 0.5% ascorbic acid was also added to both the stock solutions and the media to minimise the oxidation of the hormone. The culture dishes were incubated at 37°C for three hours and treated as before (Chapter Three Experiment 3b) to determine the incorporation of radioactivity.

4.3.2(b). Results.

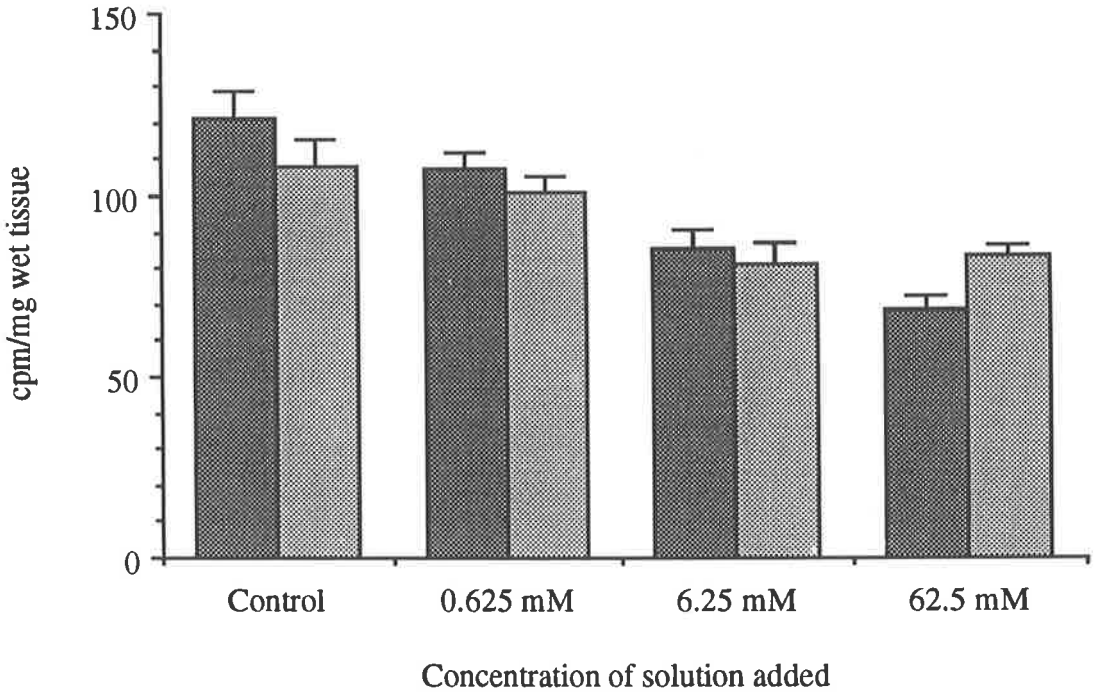


Figure 4.2. The effect of various levels of noradrenaline ■ and adrenaline ▨ in culture media on DNA synthesis *in vitro*.
Error bars indicate the standard error of the mean.

Figure 4.2 shows that at all doses of noradrenaline and adrenaline, there was a reduction in the amount of [^{3}H]thymidine incorporated and therefore in the rate of DNA synthesis in skin strips under the *in vitro* culture conditions. For both catecholamines, the reduction was not significant at the lowest dose when compared with the control, however at all other doses the depression of DNA synthesis was significant. The variability in the rate of synthesis was

marked within each treatment group, a problem which we have not been able to overcome in this laboratory. This variability could be responsible for masking any slight depressant effect the lower doses of catecholamines could potentially elicit.

4.3.3. Discussion: Experiments 4a and 4b.

The evidence of Experiments 4a and 4b were regarded as significant preliminary observations to suggest a potent effect of exogenous catecholamines on DNA synthesis, and cell division. The following experiments were designed to investigate whether physiological levels of noradrenaline were sufficient to reproduce these effects *in vivo*.

Unilateral removal of portions of the sympathetic nervous system has been used by various authors, as described in Chapter One. A similar approach was adopted here, and two alternative methods of sympathectomy were utilised.

4.4. Chemical sympathectomy.

Chemical sympathectomy of foetal lambs has been achieved by Alexander and Stevens (1980) using 6-hydroxy-dopamine (6-OH dopamine). Foldes and James (1981) showed that *in vitro* uptake of [^{3}H]noradrenaline was sensitive to 6-OH dopamine. The following brief experiments were designed to determine the short and long term effects of the sympathectomising agent, 6-OH dopamine, on cell division rate in wool follicles. Experiments were undertaken to establish the effectiveness of localised sympathectomy achieved by this method, using a monoamine histofluorescence technique similar to that used by Alexander and Stevens (1980).

4.4.1. Experiment 4c.

Aim: To determine whether endogenously-produced levels of noradrenaline can reduce cell division rates *in vivo*.

4.4.1. Experimental Procedure.

4.4.1(a). Experiment 4c (i).

Three sheep (CS1, CS2 and CS3) were injected with five intradermal doses of 6-OH dopamine (0, 50, 100, 250, 500 μg in 0.1 ml of saline) according to the pattern in Figure 4.3. Biopsies were taken from the sites two, four and six hours later from sheep CS1, CS2 and CS3, respectively. The biopsies were taken two hours after injections of colchicine (50 μg) and treated as per the method of Hynd *et al.* (1986).

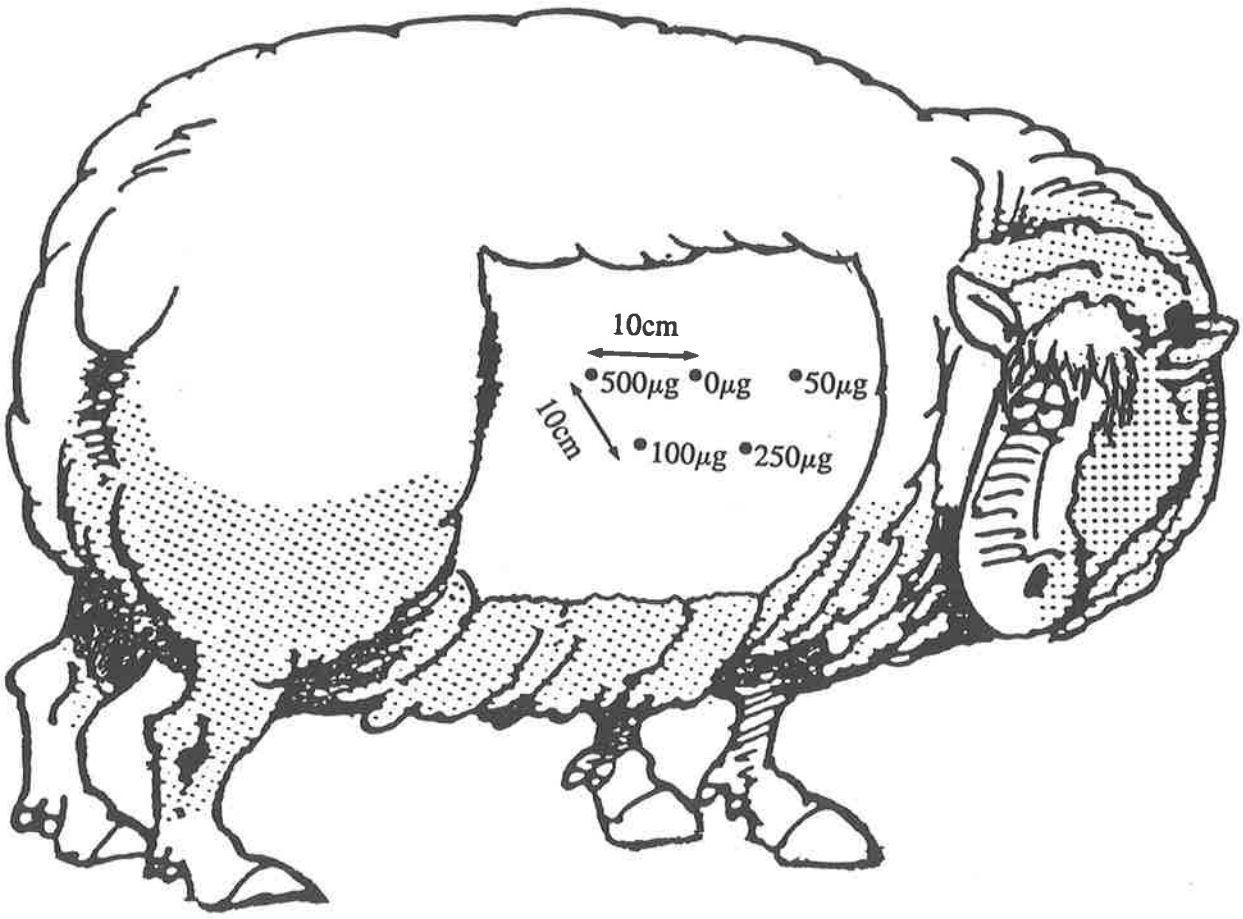


Figure 4.3. The site and dose of intradermal injections of 6-OH dopamine. Adapted from Ball (1979).

4.4.1(c). Results.

Table 4.2 The average rate of cell division in wool follicles two, four and six hours after local administration of 6-OH dopamine at four different dose levels.

	Sheep CS1	Sheep CS2	Sheep CS3
Duration of treatment	2 hours	4 hours	6 hours
Control	5.23	6.21	5.49
50 μg	5.23	5.72	5.94
100 μg	3.30[a]	6.57	5.69
250 μg	3.73[a]	6.13	5.64
500 μg	4.37[a]	5.69[b]	5.98[c]

[a,b,c] Significantly different to the respective control by Kolmogorov-Smirnov test.

It is clear from Table 4.2 that 6-OH dopamine has a significant depressing effect on cell division rate over a period of two hours for intradermal doses above 50 μg. At four hours, only the 500 μg dose maintained a significantly lower rate of cell division, which suggests that the effect diminishes rapidly with time. At six hours no dose significantly depressed cell division and, in fact, the skin sample treated with the highest dose had a cell division rate slightly but significantly higher than that of the respective control. It is important to note that statistical comparisons are made within an animal, but these results suggest that endogenous noradrenaline, released under the influence of 6-OH dopamine (Weiner, 1980), causes a reduction in cell division rate shortly after injection. Depletion of nerve terminals by the sympathectomising action of 6-OH dopamine allows cell division to accelerate at the highest dose after six hours.

4.4.1(d). Experiment 4c (ii).

At 1500 hours on day one of this experiment, three sheep (CS4, CS5 and CS6) were injected with an intradermal dose of 6-OH dopamine (500 μg in 0.1 ml of saline) into a site lying over

the tenth rib on the right midside and a matching injection of saline alone in the same position on the left midside. At 1500 hours, three days later, colchicine (50 μg) was injected intradermally into the same sites, and skin biopsies removed at 1700 hours and treated as per the method of (Hynd *et al.*, 1986). Between the injection of colchicine and the collection of biopsies, the animals were housed in a shed, in which the ambient temperature ranged from 17 to 19°C.

4.4.1(e). Results.

Table 4.3. Mitotic rate in wool follicles of control and treated sites of three sheep three days after treatment with 6-OH dopamine or saline.

Sheep Number	Saline treated site	6-OH dopamine treated site	Statistical significance†
CS4	4.46	4.32	n.s.
CS5	4.22	4.55	n.s.
CS6	5.13	4.75	n.s.
Average	4.60[a]	4.54[a]	

† According to the Kolmogorov-Smirnov test at the 5% level of significance.

[a] Overall mean for treatment and control sites, not significantly different at the 5% level using the t-test.

The results of Experiment 4c(ii) are shown in Table 4.3 and suggest that after three days, cell division rates in the wool follicles of these sheep were not affected by local treatment of the skin with 6-OH dopamine. There was no significant difference either between cell division rates in biopsies taken from opposite sides of individual animals, or the overall mean for the three animals.

4.4.1(f). Experiment 4c (iii).

At 1500 hours on day one of this experiment, three sheep (CS7, CS8 and CS9) were injected with intradermal doses of 1000 µg of 6-OH dopamine into four sites arranged over the right midside as in Figure 4.4, and matching injections of saline were made on the left midside. The dose of 6-OH dopamine was higher than those in Experiment 4c(ii), as it was suspected that the doses used in that case may have been inadequate. One week later, colchicine (50 µg) was injected into the two anterior sites on each side of the animals at 1500 hours, and biopsies were taken at 1700 hours. Into the two ventral sites on each side, intradermal doses of noradrenaline (50 µg) were injected at the same time as the colchicine treatment. During this experiment the ambient temperature of the shed in which the animals were housed ranged between 11 and 13°C.

Biopsies were removed from the posterior sites and used in an attempt to determine the level of catecholamine present in the skin, and thus establish the effectiveness of local chemical sympathectomy in the skin of sheep. These skin samples were processed as per the method of de la Torre and Surgeon (1976).

Thus, eight biopsies were removed from each animal, four to be assessed using the method of Hynd *et al.* (1986) and four to be examined for catecholamine autofluorescence. The eight biopsies consisted of two matching sets of control and 6-OH dopamine treated samples either with or without noradrenaline treatment prior to sampling.

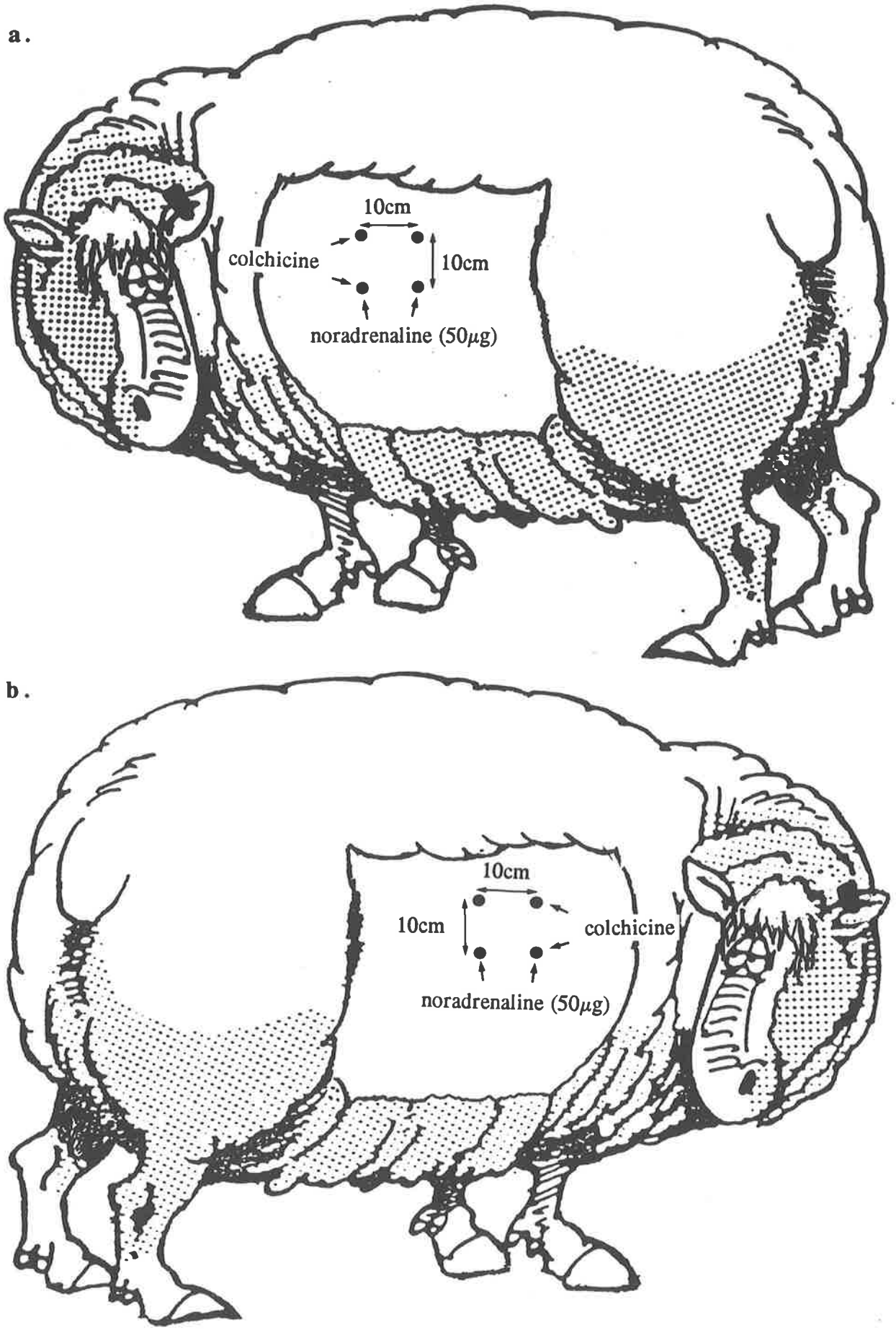


Figure 4.4. The site of intradermal injections of saline (left midside (a)), and of 6-OH dopamine (1000μg) injections (right midside (b)). Colchicine and noradrenaline were injected into sites as indicated. Adapted from Ball (1979).

4.4.1(g). Results.

Table 4.4. Mitotic rate in skin biopsies collected one week after treatment with saline (control) or a chemical sympathectomising agent (6-OH dopamine) and two hours after treatment with noradrenaline or saline.

Sheep Number	Control	6-OH dopamine	Control with noradrenaline.	6-OH dopamine, with noradrenaline.
CS7	4.62	5.46	3.40	3.95
CS8	4.16	5.60	3.35	3.03
CS9	6.15	6.24	2.79	4.36
Average	4.98[a]	5.77[a]	3.18[b]	3.78[b]

[a] Treatments significantly different at the 10% level by analysis of variance.
Treatments with superscript [b] were significantly different from those with superscript [a], but not significantly different from one another at the 5% level by analysis of variance.

Analysis of variance revealed that the effect of noradrenaline treatment was highly significant (F probability of 0.002), which strongly supports the evidence gained in Experiment 4a above, showing that a 50 μg dose of noradrenaline produced lower cell division rates than those observed in contemporary control samples. The effect of intradermal treatment of the biopsy site with 6-OH dopamine one week prior to colchicine skin sampling was significant at the 10% level (F probability of 0.094). From Table 4.4 the rate of cell division in the wool follicles was clearly increased in two out of three of these sheep. However there was no significant interaction between 6-OH dopamine treatment and noradrenaline treatment (f probability 0.795). This suggests that pre-treatment with 6-OH dopamine does not affect the response to exogenous noradrenaline treatment. Thus 6-OH dopamine affects nerve terminals only and not receptors, which makes it a potentially useful agent for these studies, although the drawbacks described below will need to be overcome.

There was a lack of an observable effect of 6-OH dopamine treatment on the amount of autofluorescence in skin samples taken from either the treated or untreated side, and subjected to the method of de la Torre and Surgeon (1976). Indeed, it proved impossible to detect a difference in catecholamine autohistofluorescence between two skin samples, one of which was treated with noradrenaline two hours prior to sampling. Either the exogenous noradrenaline may have disappeared, or the method failed to work in this tissue under the conditions imposed. Although Williams and Willis (1987) have previously used this method with success, de la Torre and Surgeon (1976) implied that producing reliable results required some expertise. Inability to demonstrate catecholamine autofluorescence during the present experiment was probably due to insufficient experience with the technique. It is also possible that the large number of amine groups associated with the tissue keratins may have obliterated the small amount of catecholamine autofluorescence present.

4.4.2. Discussion: Chemical Sympathectomy.

Experiments 4a to 4c provide strong evidence to suggest that not only do the catecholamines have the potential to reduce wool production, but that a reduction in DNA synthesis and cell division occur within a very short period of time. The results of chemical sympathectomy were inconclusive, and it proved impossible to demonstrate any difference in autohistofluorescence between treated and control sites. The following experiment was designed to improve the ability to detect successful sympathectomy, cause secretion of large quantities of endogenous noradrenaline and monitor undesirable secretion of adrenaline from the adrenal gland.

4.5. Surgical Sympathectomy.

4.5.1. Experiment 4d.

The following experiment was designed to determine the effects of endogenously released catecholamines on the rate of cell division in wool follicles, whilst providing a means of assessing the level of catecholamine secretion.

4.5.2(a). Surgical unilateral sympathectomy.

Animals were selected from a large flock of three year old Merino wethers. The average liveweight of the animals was fifty kilograms, and all exhibited a high level of face cover (Jefferies, 1964). The superior cervical ganglion was removed from the left hand side of twenty four sheep by the method of Appleton and Waites (1957) with the following modifications.

Animals were initially anaesthetised using 0.4 ml/kg sodium pentobarbitone (Ciba Geigy Australia Ltd, 5 Valetta Road, Kidman Park, South Australia, 5025) and subsequently maintained under Halothane (ICI Australia, 1 Nicholson Street, Melbourne, Victoria), with the the animal placed on the right side and rolled slightly onto its back. The head and neck were straightened and turned so that the ventral side of the head was uppermost. Using an electrosurgical cutting tool (Electrosurgical unit, model 250, Vetko, Colorado Springs, Colorado), a ten centimetre incision was made above the position of the larynx and at right angles to the trachea. By using this approach it was possible to avoid sectioning the platysma muscle, and with a great deal of care it was also possible to avoid damaging the moderately-sized blood vessels present in this region. The loss of blood into the deep regions of the wound were therefore minimised and visualising the ganglion was made much easier. Blunt dissection was also made easier by this approach, as with the neck extended, the tissues tended to fall away under tension and retraction was therefore not necessary.

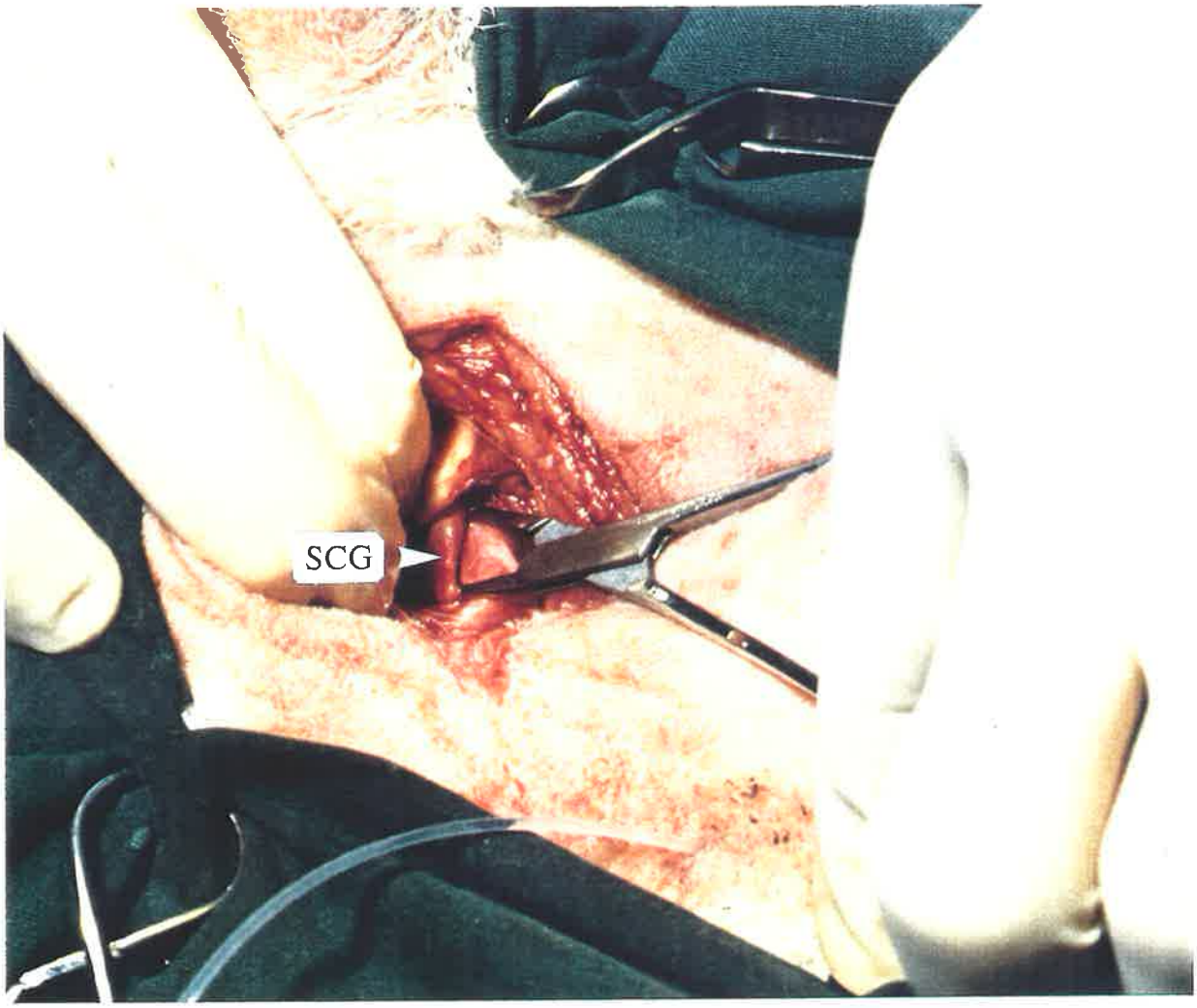


Plate 4.1. The superior cervical ganglion (SCG) of a Merino wether.

The ganglion is shown in Plate 4.1 although in this case the platysma muscle was severed and retracted, to reveal the ganglion more appropriately for photography. The ganglion was removed by carefully pulling on the branches of the nerves running into it, in preference to cutting, as it was possible to remove up to 50 mm of each nerve without fear of rupturing a blood vessel. The operation from the initial incision to closing could be completed in under thirty minutes by this technique, since it was not necessary to suture the jugulo-hyoid and platysma muscles as was the case with the approach used by Appleton and Waites (1957).

Post-operatively the animals were taken in groups of four and placed in a controlled environment room for two weeks, where they were allowed to recover and become accustomed to the conditions. The lighting regime was adjusted to the daylength at the time the animals were moved into the room. The air temperature of the room was maintained at 23 ± 1°C, at which both sympathectomised and non-sympathectomised ears assumed identical temperatures.

4.5.2(b). Experimental period.

After one week in the controlled environment room, a thermocouple (32 gauge polyethylene coated copper/constantan, Dural Plastics and Engineering, Dural NSW 2158 Aust.) was attached to each ear of the sheep using a layer of plastic skin and gauze bandage (Figure 4.5). The thermocouples were connected to a twin channel digital thermometer (Omega 871 Digital Thermometer, Omega Engineering, Stamford, Connecticut, USA) which was strapped to the animal's back. The apparatus is shown in position in Plate 4.2. Alternatively, longer thermocouple wires were suspended from the ceiling of the room and the thermocouples attached to a data logger (Squirrel, Grant Instruments (Cambridge) Ltd., Barrington, Cambridge, CB2 5Q2, England) placed on a table at a site remote from the animals. The latter device was preferable, as the recordings could be down-loaded directly to a personal computer, and it was possible to undertake the following parts of the experiment unhindered. The thermocouples were calibrated against a mercury thermometer, which was also suspended within the room to facilitate recording of the environmental temperature.

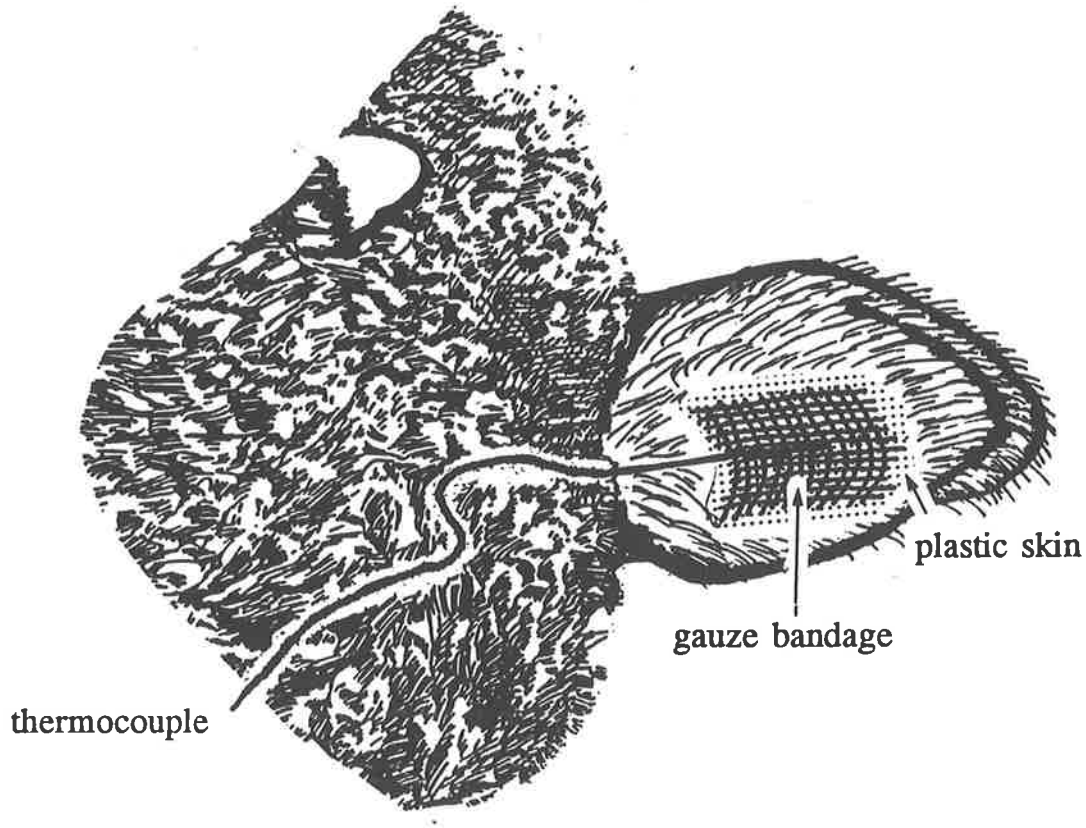


Figure 4.5. Sketch showing the method of attachment of the thermocouples to the ear of the sheep using a gauze bandage and plastic skin.

Plate 4.2. A Merino wether with a digital thermometer mounted on its back to record ear temperatures during environmental cooling.

After fixing the thermocouples to the ears, the sheep were left for a few hours to recover from the excitement involved with handling, and to allow the temperatures of the ears to stabilise. Commencing at 1200 hours the temperature of the room was lowered from 23°C to between 6 and 8°C for a period of three to four hours. The temperature of the room and of each ear of each animal was recorded every 15 minutes. After about one hour, the temperature of the room had decreased by about 13°C to around 10°C. Animals varied in their response to this change in room temperature, and in most cases, were left untouched until a difference of 10°C had been established between each ear. At this time, an intradermal colchicine injection (50μg) was made

into both cheeks of the animal. The site of injections is shown by the black circle drawn on the cheek of the sheep in Plate 4.3. Two hours later skin biopsies were removed from each cheek and processed as per the method of Hynd *et al.* (1986). A few animals showed no cooling response within three hours of lowering the environmental temperature; in these sheep, injections were administered at three hours and biopsies collected two hours later.

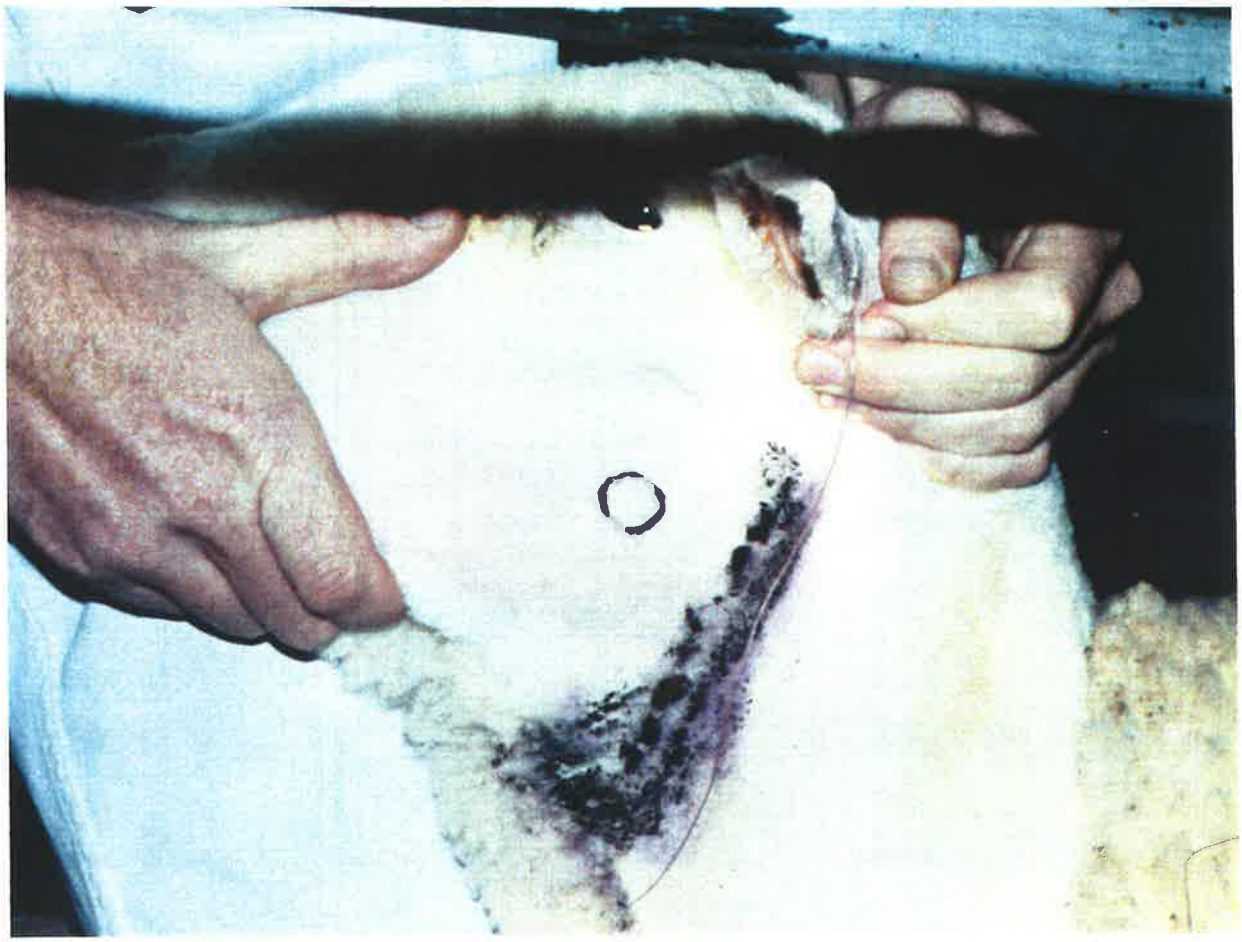


Plate 4.3. The colchicine injection site marked on the cheek of a Merino wether. Also clearly visible is the suture line which delineates the site of entry to the superior cervical ganglion.

4.5.2(c). Results.

Unilateral sympathectomy of the head region and subsequent lowering of the environmental temperature brought about a reduction in the rate of cell division in wool follicles of the cheek skin of a number of the sheep. The measured response of the ears varied between the animals, and some examples of the different ear responses are recorded below. In half of the animals the surface temperature of the ear on the unoperated side was lowered with respect to the

sympathectomised ear, although the time of onset of the cooling response and therefore the time of colchicine injection varied. To show the average pattern of response in Figure 4.6, the time at which colchicine was injected has been taken as zero minutes, due to the irregular times at which the cooling response commenced. The large standard error of the room temperature prior to the injection of colchicine reflects the variability of injection times between animals, and not fluctuations in the rate of cooling of the room, which was a very smooth process.

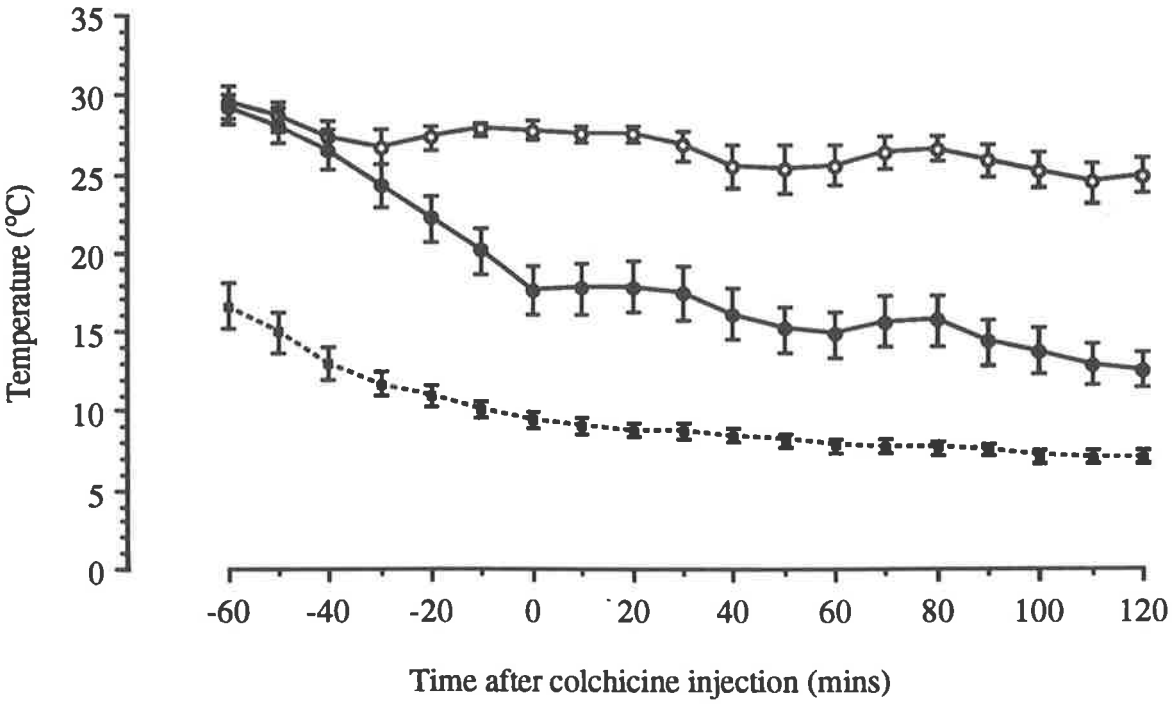


Figure 4.6. The average response of the sympathectomised (○) and unoperated (●) ears to lowered environmental temperature (·····●·····). (Error bars indicate the standard error of the mean.)

All animals used in the experiment consumed all of the feed offered. When recording ear surface temperatures, injecting colchicine and finally collecting the biopsies, some animals became very excited. This was reflected as a drop in the surface temperature of the sympathectomised ear.

The cell division rates measured in cheek skin after two hours of treatment with colchicine are shown in Table 4.5. The sheep have been split into two groups; those which responded to

cooling in the expected manner and those which did not. Examples of these responses are provided below.

Table 4.5. Cell division rate in skin biopsies collected from Sympathectomised (Sympath.) and Unoperated (Unoperated) cheek sites after two hours of treatment with colchicine, and the relative difference between cell division rate in the two cheek sites (% different) in animals which exhibited the expected cooling of the unoperated ear (Expected Response) and those which did not (Unexpected Response).

Expected Response				Unexpected Response			
Sheep No.	Sympath.	Unoperated	% different	Sheep No.	Sympath.	Unoperated	% different
SS24	3.89[a]	3.08[a]	-20.8	SS14	4.35	4.01	-7.8
SS1	4.71[a]	3.79[a]	-19.5	SS22	4.53	4.20	-7.3
SS19	3.54	2.97	-16.1	SS23	5.43	5.36	-1.3
SS16	3.26	2.74	-16.0	SS17	4.15	4.11	-0.9
SS20	4.26	3.80	-10.8	SS5	3.41	3.40	-0.3
SS3	4.63	4.25	-8.2	SS15	3.80	3.83	0.8
SS18	4.04	3.73	-7.7	SS7	4.53	4.59	1.3
SS13	3.11	3.07	-1.3	SS6	3.52	3.57	1.4
SS4	3.58	3.97	10.9	SS2	3.89[a]	4.67[a]	20.1
Average	**3.89[b]**	**3.49[b]**		**Average**	**4.18**	**4.19**	
SD	**0.57**	**0.53**		**SD**	**0.62**	**0.61**	

[a] Significant difference between the cheek sites according to the Kolmogorov-Smirnov test (5% level).

[b] Significantly different according to a paired t-test.

For reasons discussed in Appendix Two, the mean cell division rates of a number of biopsy samples collected using the colchicine technique is found to be normally distributed, and it is thus possible to use parametric statistics. Since the sympathectomised and unoperated biopsies were matched pairs, the results were analysed using a paired t-test and found to be significantly different ($p < 0.026$). The Kolmogorov-Smirnov test revealed a significant difference in cell division rate in biopsies from unoperated sites of two animals which exhibited the expected response (SS1 and SS24). These were the animals which showed the greatest reduction in cell division on the unoperated side with respect to the operated side. One animal which exhibited an unexpected response (SS2), also exhibited a significant difference in cell division rate, although in this case the highest rate of cell division was determined in the unoperated ear. In this experiment the paired t-test was therefore slightly more sensitive than the Kolmogorov-Smirnov test.

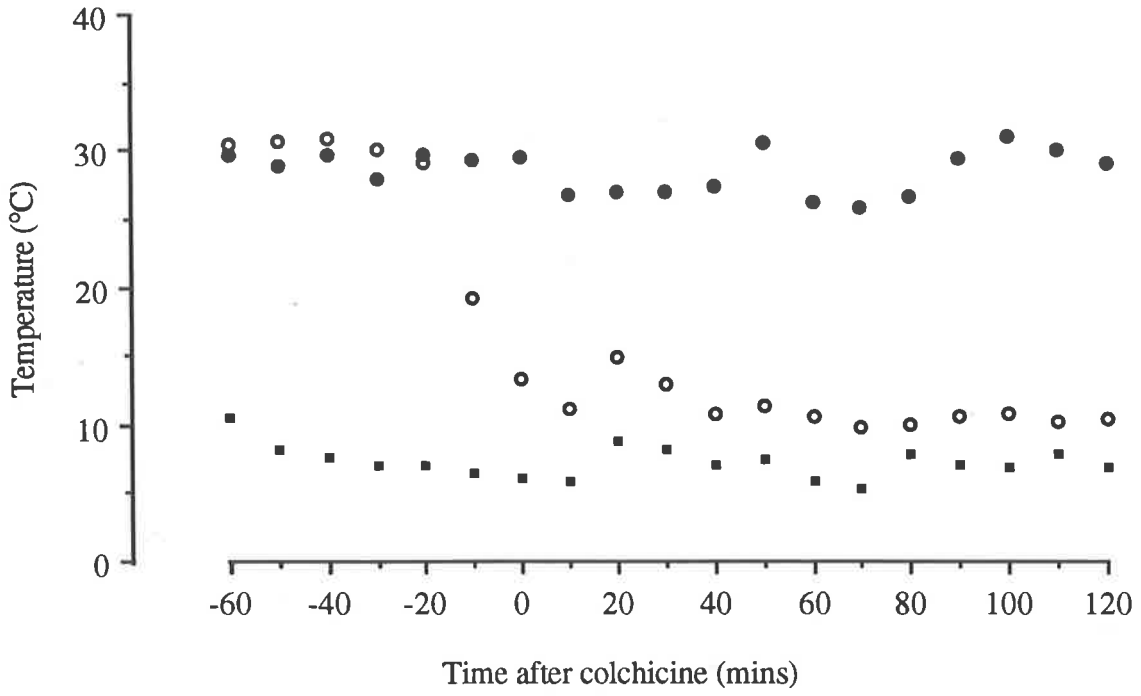


Figure 4.7. An example of the expected response to lowered environmental temperature (▪), exhibited by animal SS18. This type of response was characterised by a clear separation of the temperatures of the sympathectomised (●) and unoperated (o) ears over the entire period of colchicine arrest.

An expected response has been displayed graphically in Figure 4.7. This graph shows the temperatures of the room, sympathectomised and unoperated ears at ten minute intervals for sixty minutes prior to injection of colchicine and 120 minutes thereafter. This was the ideal response, and all sheep in this group in Table 4.5 showed this clear separation.

Unexpected responses included periods of increased temperature of the ear on the unoperated side after colchicine injection, periods of cooling of the sympathectomised ear and no cooling in either ear. Some examples of the unexpected responses are provided below, as they are more informative than an overall average of the type shown in Figure 4.6 above.

Despite the fact that the temperature of the room was evenly lowered to 6°C in most cases with air circulating freely, there were some animals which did not exhibit any large cooling response in the unoperated ear during the entire period of colchichine treatment. However all these animals exhibited positive signs of successful sympathectomy, including relaxation of the lower eyelid and the nictitating membrane (Appleton and Waites, 1957). Retesting of the animals in a cold environment one day after skin sampling, produced a clear separation in the temperature of the ears. On the occasion of sampling, these animals were obviously less sensitive to the environmental temperature, perhaps as a result of excess metabolic heat after feeding, or increased blood pressure for some reason. Animal SS14 provides an example of such a response which is illustrated in Figure 4.8. A slight cooling response commenced after 90 minutes in the cold environment at the time of colchicine injection, however, post-injection the cooling effect vanished and did not resume between injection and two hours later when the skin samples were removed. Whatever the reason for the lack of a response in ear temperature, cell division rates determined from these sheep were not consistently lower on one side with respect to the other.

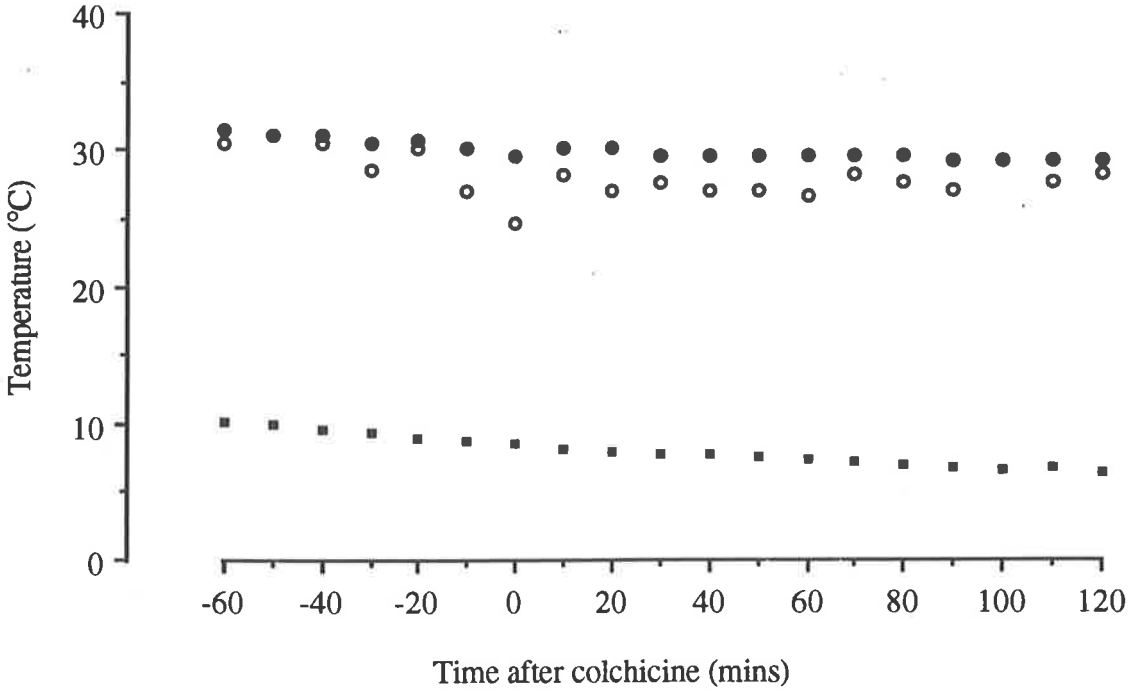


Figure 4.8. An unexpected response exhibited by animal SS14 during a period of cold environmental temperature (▪), showing a lack of separation of the temperatures of the sympathectomised (●) and unoperated (o) ears over the entire period of colchicine arrest.

Sheep SS22 (Figure 4.9) exhibited distinct differences between the temperatures of sympathectomised and unoperated ears, which were not maintained for the entire period of colchicine treatment. This was another example of an unexpected response, where the temperature of the sympathectomised ear fell on two occasions during the experiment. In this response, it is likely that there was some secretion of adrenaline from the adrenal gland with resultant vasoconstriction and cooling of the ear on the operated side. This release of adrenaline was possibly due to an increased human presence in the controlled environment room or excitement due to the cooling of the room.

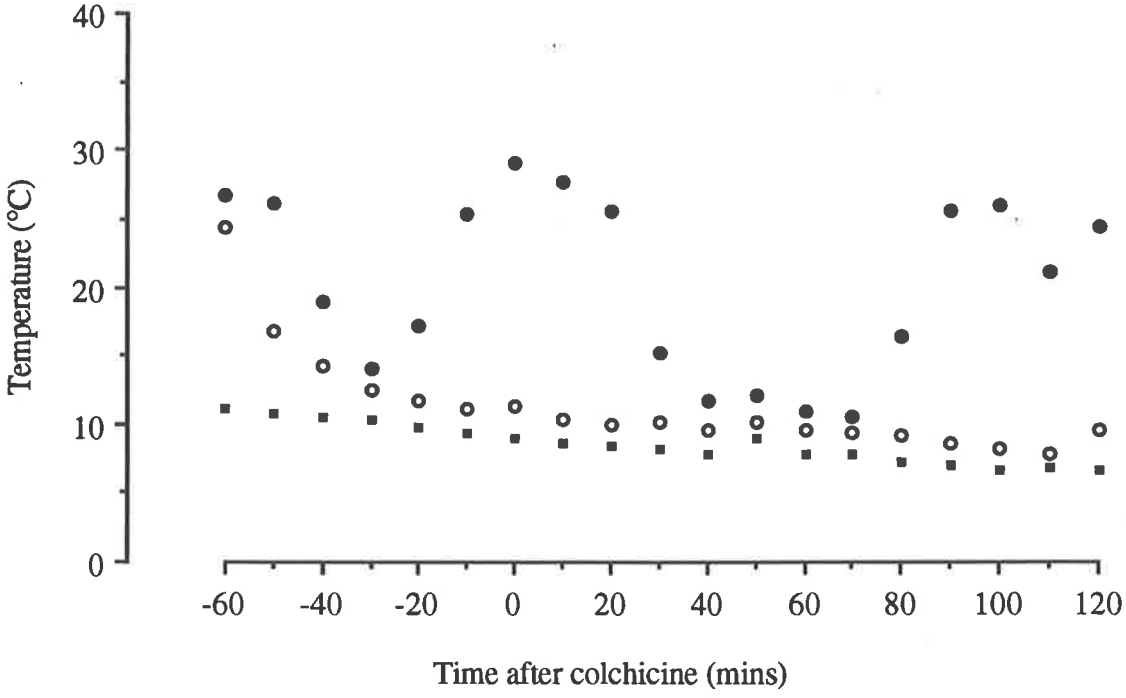


Figure 4.9. The response exhibited by animal SS22, an example which suggested that adrenaline was being released from the adrenal gland. This type of response was characterised by a decrease in the temperature of the sympathectomised ear (•) during the period of colchicine arrest. (Environmental temperature ▪ , unoperated ear o)

In sheep SS8 the accidental rupture of a large blood vessel, while attempting to remove the superior cervical ganglion resulted in abortion of the operation. This animal was subsequently used as a sham-operated control to detect whether the operation *per se* would affect wool follicle mitotic rate on one side of the animal with respect to the other. When the temperature of the room was lowered, both ears of this animal demonstrated a parallel cooling response as shown in Figure 4.10. There was no significant difference in the rate of cell division of skin samples taken from opposite cheeks at the 5% level using the Kolmogorov-Smirnov test. The results were not included with those in the unexpected response group in Table 4.5 because this animal was not correctly sympathectomised. The mean cell division rates were 4.76 on the sham-sympathectomised side and 4.66 on the operated side.

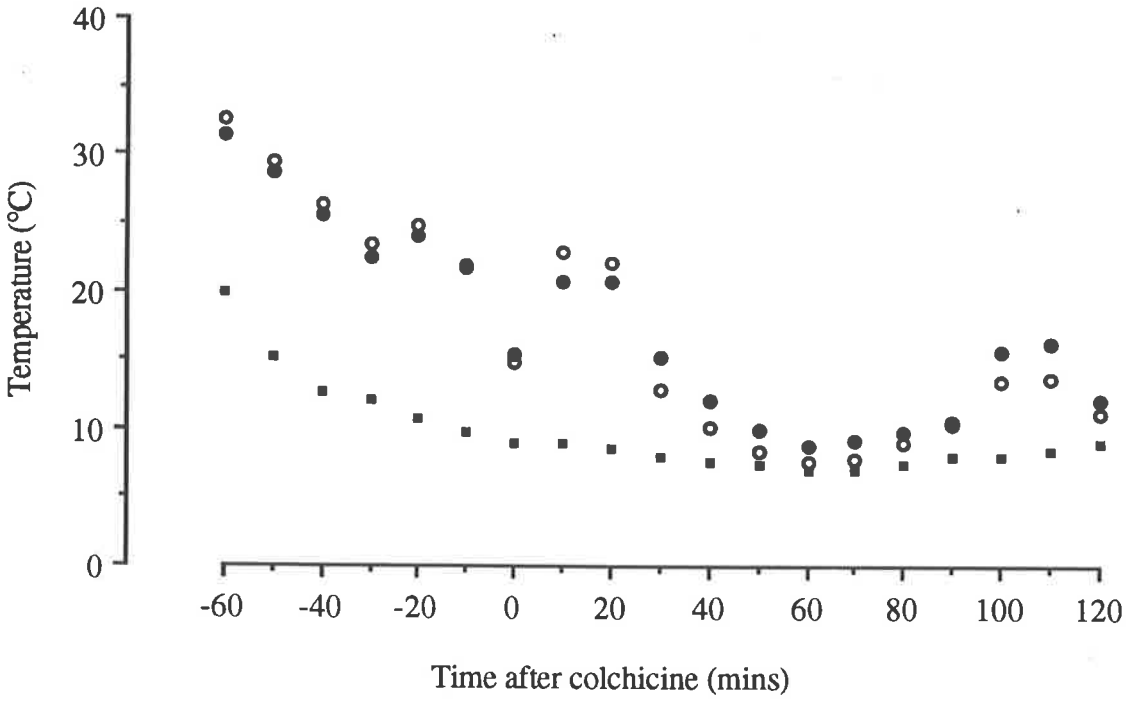


Figure 4.10. Ear temperature responses in an animal (SS8) which underwent a sham operation (Sham operated ear ● , Unoperated ear ○ , Environmental temperature ▪), showing cooling of both ears over the entire period of colchicine arrest.

In total, twenty-four animals were subjected to cooling. One pair of biopsies, from animal SS21, were spoilt during processing. This animal exhibited a cooling response similar to that shown in Figure 4.9. Animals SS9, SS10, SS11, and SS12, were excluded from the study due to a malfunction of the air conditioning unit in the controlled environment room. Although the temperature in the room only increased slightly, condensation in the cooling fins had frozen solid and the circulating fans failed to move air around the room. In the still air, the effectiveness of the cooling was greatly reduced and the ear temperatures of the animals recovered rapidly to pre-cooling levels. Attempts to restore the air conditioning unit within two hours after the injection of colchicine failed, and biopsy samples were not collected from these animals.

4.5. Discussion.

The balance of the evidence presented above shows that changes in catecholamine levels do have the capacity to influence cell division in the follicle bulb within a short period of time.

From the results of Experiments 4a, 4b and 4c(iii) it was clear that pharmacological doses of catecholamines possess the capacity to rapidly lower the rate of cell division and DNA synthesis in the wool follicle. These results are in accordance with much of the work reviewed in Chapter One with respect to catecholamines. Since Experiment 4b was an *in vitro* experiment, it follows that the effect of the catecholamines on DNA synthesis, at least, occurs independently of the effect these hormones have on vascular tissues. A direct antimitotic effect of noradrenaline may be responsible (Powell *et al.* 1971) and further work in this area will help to resolve the debate over whether the effect is extravascular. Such work is outside of the scope of the present investigations however, as the primary aim of the work was to determine how rapidly, rather than how, the effects take place. It could be that catecholamines have some influence on all of the compartments involved in wool fibre production on a local basis within the skin, both independently of, and also as a direct consequence of, vasoconstriction.

Leading up to the first attempt at using chemical sympathectomy on a small localised area, 6-OH dopamine itself was found to significantly reduce cell division rates (Experiment 4c(i)). The lowered cell division rates in the samples taken two hours after intradermal injection were most likely to have been a response to the sympathomimetic action of 6-OH dopamine, which is caused by a release of noradrenaline from nerve terminals in response to the uptake of 6-OH dopamine (Weiner, 1980). Comparisons between the duration of the treatment revealed that although 6-OH dopamine initially reduced the level of cell division, a rapid recovery was evident after four and six hours. The short period of time taken for mitotic rate to recover from a dose of 6-OH dopamine in Experiment 4c(i), shows that the effect of 6-OH dopamine in the subsequent experiments was unlikely to be a direct consequence of the sympathomimetic action of the drug.

It would appear from Experiment 4c(ii) that chemical sympathectomy using 6-OH dopamine had no significant effect on cell division rate. However, the ambient temperature for the duration of this experiment was at a level that may have been insufficient to produce a significant release of noradrenaline from nerve terminals in the skin (17 to 19°C). Further experimentation with chemical sympathectomy showed that this was possibly the case, and in Experiment 4c(iii) two out of three sheep demonstrated a significant increase in cell division rate on the sympathectomised side. During Experiment 4c(iii) the ambient temperature was lower (11 to 13°C) but may not have been sufficient to produce an adequate level of noradrenaline secretion to reduce cell division rates in all three sheep. The results of Experiment 4c(iii) also showed that the mechanism by which cell division responds to exogenous treatment with catecholamines is not impaired by pre-treatment with 6-OH dopamine.

The response of the control skin sites of the chemically-sympathectomised animals to cold could be expected to be as variable as the response of the ears in Experiment 4d. Since it was not possible to demonstrate whether these animals had been successfully sympathectomised, the lack of difference in cell division rates observed in sheep CS9 may also be explained by ineffective local sympathectomy. The modified glyoxylic acid method developed by de la Torre and Surgeon (1976), had been successfully used by Alexander and Stevens (1980) in ovine tissues, and was attempted in the present work. Williams and Willis (1987) used the Falck-Hillarp fluorescence histochemical method, which may have been better suited to skin tissues of sheep. The ease of superior cervical ganglionectomy and subsequent physical indicators of the success of this operation (relaxation of the nictitating membrane) were the reasons that chemical sympathectomy was abandoned in favour of surgical sympathectomy.

Although the numbers of animals studied by chemical sympathectomy were small, when all of the results were considered together it is possible to say that the procedure showed some promise in its ability to cause an increase in cell division rate with respect to control sites. It is possible that failure to demonstrate a similar response in all animals could be either an effect of inadequate cooling, or lack of effective chemical sympathectomy.

Using surgical sympathectomy and the measurement of the surface temperature of the ear it was necessary to establish a set of criteria to describe the response of any given animal in order to test the hypothesis that endogenous levels of noradrenaline would affect cell division rate. The different types of response observed throughout the period of colchicine arrest have been described above. Essentially the temperature response was used as a bioassay for endogenous noradrenaline secretion. Setchell and Waites (1962) have previously shown that ear temperature (after sympathectomy) responds in a graded or dose-dependent manner to infusion of different levels of adrenaline. A decline in the temperature of the sympathectomised ear was considered to be an accurate indicator of adrenaline release, and the cheek skin on the unoperated side of the animals which exhibited such a response would have been exposed to a complicated mixture of the two catecholamines. Ferguson *et al.* (1965) suggested that endogenous adrenaline does not affect wool growth, and further examination of animals which demonstrate this type of response may assist in understanding whether endogenous release of adrenaline affects cell division. Although there were inadequate numbers of these animals in Experiment 4d, the results of the *in vitro* experiment suggest that adrenaline may have similar effects to noradrenaline.

The animals were grouped into two major groups according to the response of their ears, those in which the two biopsy sites were exposed to different amounts of noradrenaline, and those for which this was not true. Of the animals which exhibited a decline in ear temperature on the unoperated side, there was only 1 sheep (Sheep SS4) which did not yield results consistent with the hypothesis, i.e. a lower cell division rate in the unoperated site. The other animals exhibited lower cell division rates on either side with respect to the other.

It is important to note that two hours of endogenous release of noradrenaline elicited only a small response in cell division in a number of cases, the greatest difference being about twenty percent, in animal SS24. However, a larger or more sustained release over longer periods of time would be expected to produce a larger influence on cell division, based on the results of other experiments of this chapter. Thus it is now possible to say that the fluctuations in cell division rate observed in circadian experiments may be caused by the release of noradrenaline.

There is a large amount of evidence to suggest that cold reduces wool growth rate (Doney and Griffiths, 1967; Slee and Ryder, 1967; Downes and Hutchinson, 1969; Jolly and Lyne, 1970; Lyne *et al.*, 1970), and from the results of the present chapter, it is likely that this effect is due to the influence of catecholamines. The experiments of Wodzicka-Tomaszewska and Bigham (1968) showed that covering closely-clipped patches, with bags of wool, reduced the seasonal rhythm of wool growth that has often been observed when using the "tattooed-patch" method. Local cooling of the closely clipped tattooed patch would cause the release of noradrenaline, a reduction in cell division rate and therefore a reduced rate of wool growth. When the patch remained insulated there would be less noradrenaline released. Bigham (1974) studied this further and found that the ratio of weight of wool clipped from a midside tattooed patch to weight of the entire fleece varied depending on whether the entire sheep was shorn or only the patch clipped. This would complicate the response of the clipped patch to cold on the animals studied by Bigham (1974), as the whole body would respond to cold and could no longer employ regional heterothermy of the patch to retain heat. From this work and the results presented here, it is likely that the seasonal rhythm in wool growth rate measured in many breeds is, at least in part, an artifact of the technique used to measure it. Once again the analogous nature of seasonal and circadian rhythms in wool growth rate is evident, circadian rhythms in cell division rate may similarly be exacerbated by the method used to measure the phenomenon. This concept is examined further in the following chapter.

The evidence presented here is the first to show that catecholamines have a suppressing effect on cell division in wool follicles and to demonstrate that this effect can be produced within time periods of less than a few hours *in vivo*. However, the present work is not the first to show an effect of the catecholamines on cell division rate in epithelial tissues. Bullough (1962, 1965) reviewed literature which provided evidence to suggest that mitotic rates of certain tissues (primarily of rodents) were strongly influenced by the presence of catecholamines. Related studies were reviewed earlier in the present report, and there is a considerable amount of evidence to support the work of Bullough (1962, 1965). It should also be noted that Bullough (1962, 1965) presented evidence which suggested that mitosis in active hair bulbs remained unaffected by large doses of adrenaline. This is in contrast to observations in the current thesis,

and suggests that the growth of wool is not controlled by identical mechanisms to those which control hair. Powell *et al.* (1971) have also demonstrated an inhibitory effect of noradrenaline on cell division, and now mitotic rate in wool follicles can be added to this list.

Ferguson (1949) was the first to show an effect of unilateral sympathectomy on the rate of wool growth in sheep, however he put forward the hypothesis that this effect was due to changes in blood flow to the skin. Cunningham *et al.* (1979) and Williams and Willis (1987), put forward an alternative hypothesis, that the effects may not be directly related to blood flow. Extravascular sites showing evidence of catecholamine histofluorescence, were touted as the sites responsible for this (Williams and Willis, 1987). On the other hand, Foldes and James (1981) provided evidence which failed to support the conclusions of Cunningham *et al.* (1979) as discussed in Chapter One (Section 1.2.1).

Very little has been reported on the effects of blood flow *per se* on wool growth, other than implication of blood flow changes as a result of the effects of temperature. Setchell and Waites (1965) provide some measurements of the blood flow in the skin of different areas of the body of Merino sheep, including portions of the head and the ear skin. Blood flow in the ear was found to be highly variable in these experiments (Setchell and Waites, 1965), which, considered alongside earlier work by the same authors (Setchell and Waites, 1962), would most likely be due to fluctuations in catecholamine levels. The surface temperature of the ears is therefore a precise indirect indicator of the level of catecholamines present in the region.

Dellow *et al.* (1988) found that infusing 0.4 ml/min, of a solution containing 2.5 pg/ml of noradrenaline, into the deep circumflex iliac artery for a twenty minute period stopped venous flow from the region supplied by this vessel. It is difficult to reconcile an injection of 100 µg of noradrenaline into the dermis of the midside, with infusion of 20 pg of the drug into a more diffuse region of the body over a twenty minute period. It is likely that the highest doses of noradrenaline administered in Experiments 4a and 4c are extreme. However, these were preliminary experiments based on the work of Cunningham *et al.* (1979), who treated sites with similar doses. A much more satisfactory approach than either of these methods is the physiological level of noradrenaline achieved by secretion from the sympathetic nervous system

in response to cold, as in the last experiment of this chapter. Systemic infusion as a route of administration would be likely to bias any observations towards vascular influences, and intradermal injections could be more likely to bias the effects towards extravascular sites, whereas the natural release of physiological amounts of noradrenaline into the tissues would act upon the receptive organs in the appropriate manner. Further experimentation with chemical sympathectomy may provide an even more subtle and less invasive technique to examine the effects of noradrenaline.

In conclusion, the experiments described in this chapter showed that mitotic activity in the wool follicle is reduced by exogenous catecholamines *in vivo* and *in vitro*, and that this could be reproduced by stimulating the release of endogenous noradrenaline. It is not apparent whether the results of surgical and chemical sympathectomy are an indirect result of blood flow, although the effects of the catecholamines *in vitro* suggest that these neurohormones may, at least in part, be directly responsible for changes in cell division in the wool follicle.

Chapter Five.

Reassessment of circadian rhythms in the rate of cell division in wool follicles.

5.1. Introduction.

It has been established by earlier work (Hynd *et al.*, 1986; Scobie and Hynd, 1987) and in Chapter Two of the present work that the rate of cell division in wool follicles fluctuates throughout the day. However, some uncertainty remains as to whether the mechanism responsible could be a natural circadian rhythm, or whether it was imposed by the intermittent feeding schedule, or stress created by the sampling regime itself. Critical examination of the experiment carried out in Chapter Two revealed that either of the latter two reasons could have been responsible for the randomly fluctuating rate of arrested mitoses observed. Thus it was considered possible that a superimposed fluctuation due to sampling was masking any circadian rhythm present.

Although stress steroid levels increase in sheep during handling, the experiments of Chapter Three showed that steroid levels are unlikely to cause rapid changes in the rate of cell division. The experiments of Chapter Four on the other hand showed that the catecholamine stress hormones rapidly lower cell division rate *in vivo* and the rate of DNA synthesis *in vitro*. It was also shown that the effect on cell division rate could be reproduced by the release of physiological levels of noradrenaline *in vivo*. *In vitro* experiments showed that adrenaline can have a similar effect to noradrenaline on DNA synthesis and *in vivo* experiments suggest that the two catecholamines have similar effects on cell division rates as well.

The observed effects of the catecholamines and suspected circadian changes in the levels of the neurohormones from Tindal *et al.* (1985) (Chapter Two, Section 2.4.2), could explain the circadian rhythm detected by Hynd *et al.* (1986) and Scobie and Hynd (1987). It was therefore perceived as important to re-address the question of whether wool follicles exhibit any circadian change in the rate of cell division. It was essential to keep in mind that adrenaline was likely to be released as a result of any undue excitement, as it was feasible that this could produce erroneous results. To keep the level of stress to a minimum, and to ensure that the level of excitement did not become cumulative during a single day of sampling, the second of the following experiments was designed to produce a skin sample corresponding to every second hour of the day by spreading the sampling events over a fourteen day period.

It became evident in Chapter Three that cell division rate was not affected by cortisol levels, however, Foldes *et al.* (1985) had previously shown that depilation force varied throughout the day and suggested that this might be associated with circadian changes in the level of cortisol. It is not entirely clear which compartment of wool production the depilation force measurement is dependent upon, although if we consider that keratinisation might be more important than cell division in determining depilation force then the suggested causative relationship (Foldes *et al.* 1985) could hold irrespective of changes in cell division rate. Also, if cortisol were able to affect keratinisation rapidly, without influencing either cell division rate or the rate of DNA synthesis in cells, then a circadian fluctuation in total wool growth rate might occur without a similar change in DNA synthesis or mitosis.

The following experiments were designed to further examine the rate of cell division in wool follicles of the midside region. Lincoln sheep were used in the experiment since they have a rapid wool growth rate, and therefore a rapid mitotic rate, which on the basis of previous work could be expected to show the greatest fluctuations in cell division.

5.2. Experimental Procedure.

Six mature non-pregnant Lincoln ewes were housed in individual pens in a controlled environment room in which the lights were automatically switched on at 0600 hours and off again at 1800 hours. The temperature was maintained at 23 ± 2°C. The animals were fed 800g of a pelleted ration and 400g of lucerne chaff each at 0900 hours, and the room was cleaned at this time. Although it was originally intended to impose a restricted feeding period of two or three hours on these ewes, it soon became evident that despite the large quantity of feed offered, these animals consumed all of the diet within four hours and at least 80% of it within three hours. This was checked daily throughout the duration of the experiment.

5.2.1. Experiment 5a.

The following experiment was conducted to determine the length growth rate of wool at various sites across the midside of a Lincoln ewe.

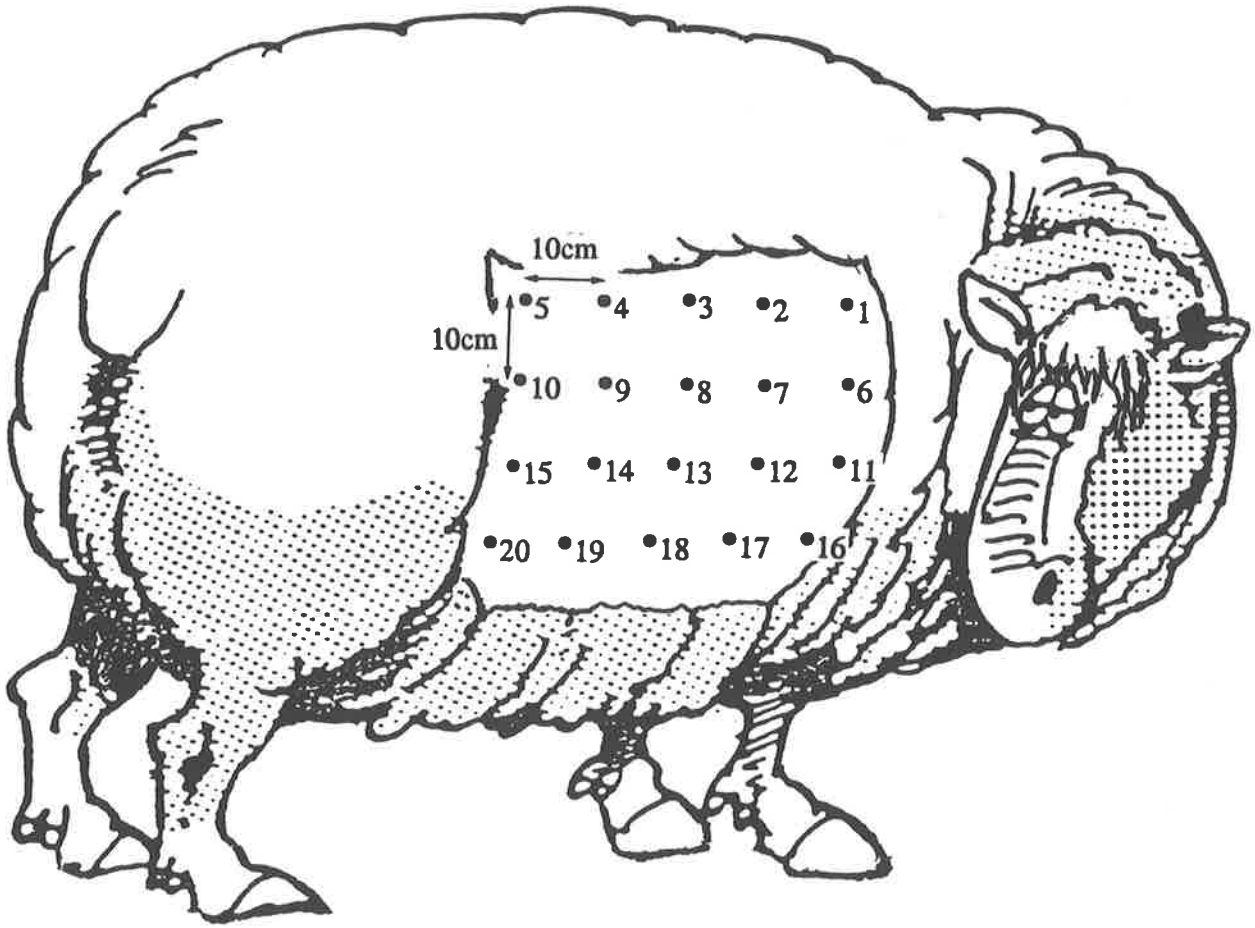


Figure 5.1. The sites of intradermal injections of [^{35}S]cysteine into the midside of Lincoln ewe L1. Adapted from Ball (1979).

At 1600 hours on day 1 of the first experimental period [^{35}S]cysteine (0.5 μCi in 0.1 ml of saline) was injected intradermally into twenty sites over the right midside of one ewe chosen at random from the six (L1). The injection sites were delineated by small circles marked using a felt tipped pen prior to injections, and the spatial arrangement of the sites were as shown in Figure 5.1. Seven days later a second injection was made into each of the twenty sites at 1600 hours.

After a further week, fibres were clipped from all of the sites, and processed and set out in cassettes for autoradiography according to the method of Downes *et al.* (1964) using "Agfa Structurix" autoradiography films (4.5 x 17in, D7pFW, Agfa-Gevaert, Belgium). To measure

the length of fibre grown between the two injections, the image of the fibre thus obtained was enlarged using the autoradiograph as an overhead transparency on an overhead projector. This device was carefully calibrated using an accurate eyepiece micrometer, which revealed that the magnification achieved was 18.95 fold. The device was essentially a "scaled-up" version of a projecting microscope and increased the speed at which fibre length measurements could be made. Tracings of the resultant images were made on blank paper and the distance between each radioactive hotspot measured with a map reading wheel (Minerva, Switzerland). The average length of fibre grown between the first and second points of injection were recorded for thirty fibres from each sample site.

5.2.2. Experiment 5b.

The following experiment was conducted to determine whether cell division rate at different times of the day were significantly different, when the level of handling stress imposed on the animals was kept to a minimum.

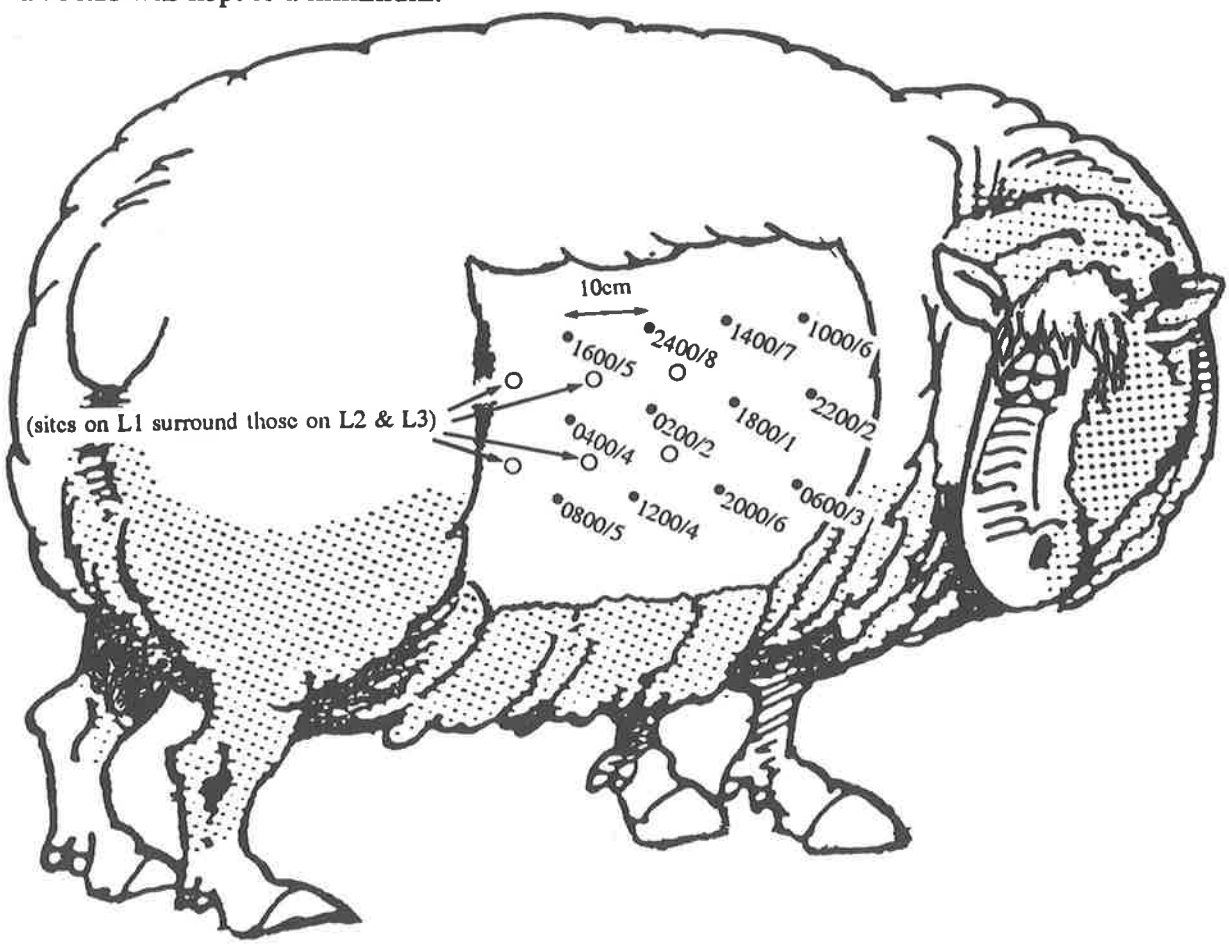


Figure 2.2. The time of day, and the day of sampling (represented as time/day) of biopsies collected from Lincoln ewes. Adapted from Ball (1979).

After a further five weeks under the same conditions as in Experiment 5a, the six ewes were used in a skin sampling experiment designed to minimize the cumulative amount of stress associated with the collection of a number of consecutive skin biopsies. In total, twelve skin samples were taken from each ewe, one corresponding to every second hour of the day, spaced over an eight day interval. The time/day/site relationship of the samples has been illustrated in Figure 5.2. The optimum dose rate of colchicine was determined previously (100 μg, see Appendix One) and skin samples were taken two hours after intradermal colchicine injection. Only two ewes (L2 and L3) were finally chosen for study, as they became noticeably less distressed than the other animals during sampling and the corresponding skin samples were treated according to the method of Hynd *et al.* (1986).

5.3. Results.

5.3.1. Experiment 5a.

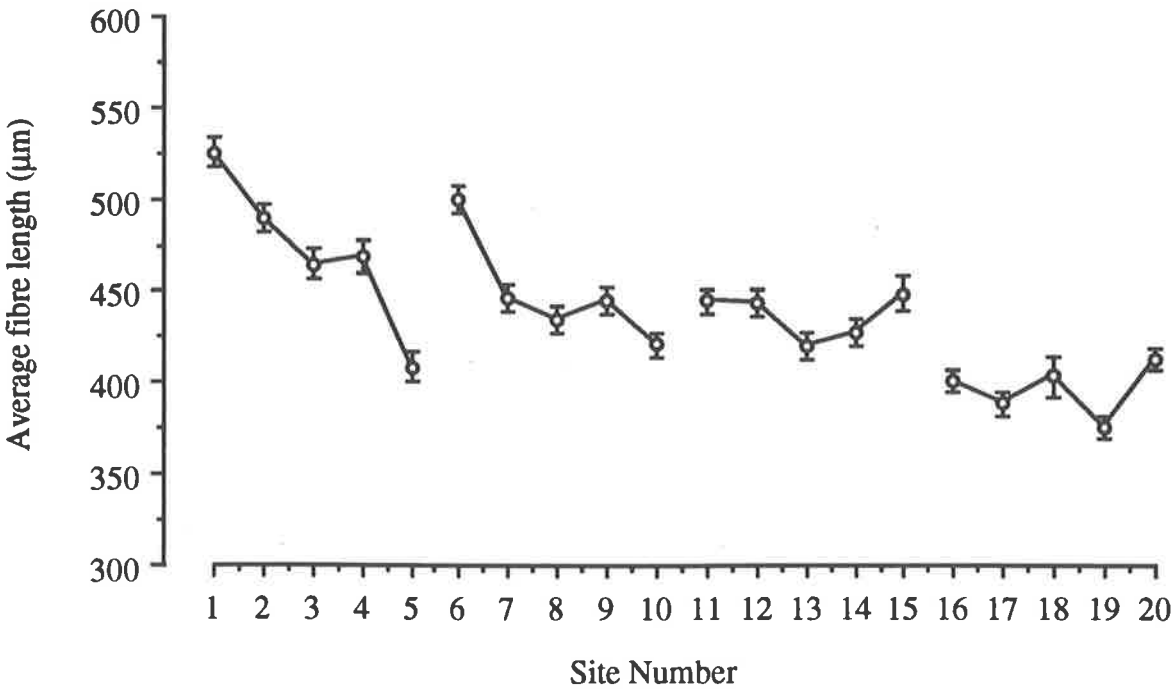


Figure 5.3. Average fibre length (μm) of thirty fibres grown over a period of one week at twenty sites on the midside of a Lincoln ewe. Error bars indicate the standard error of the mean.

It is evident from Figure 5.3 that the rate of length growth of the fibres was not uniform across the side of the animal. There was a general trend of decreasing fibre length from the anterior sites to the posterior sites and also from the dorsal to the ventral sites. The fibres from site nineteen were the shortest, at only 375 µm (sd. ± 35), whereas those from site one were were 40% longer, at 525 µm (sd. ± 45). Clearly, the rate of length growth across the side of the animals was not uniform.

5.3.2. Experiment 5b.

The cell division rate data for the two ewes have been displayed in Table 5.1, as though the samples were collected during a single twenty-four hour period. The turning points test of randomness (Kendall, 1976), revealed no significant pattern. However, from Table 5.1 cell division rate is variable, and it would appear that cell division rate decreases between 0200 and 0400 hours.

Table 5.1. The time of day, the day of sampling in parentheses, and the mean rate of cell division at the various sites for Lincoln ewes (L2 and L3).

Time of sample (day of sample)	Sheep L2	Sheep L3
0200 (2)	7.52	8.17
0400 (4)	4.79	6.26
0600 (3)	6.92	8.06
0800 (5)	6.08	7.01
1000 (6)	6.25	6.25
1200 (4)	5.93	6.88
1400 (7)	6.88	7.09
1600 (5)	6.75	8.00
1800 (1)	7.33	7.64
2000 (6)	6.44	7.57
2200 (2)	6.24	8.19
2400 (8)	6.23	7.18

5.4. Discussion.

Both cell division rate (Hynd *et al.*, 1986) and fibre length growth rate (Langlands and Wheeler, 1968; Downes *et al.*, 1964) have previously been used as indicators of total wool growth rate. The experiments outlined in this chapter have combined both of these techniques to determine whether a circadian rhythm in the rate of wool growth is apparent. Under the conditions imposed in the present experiment, the rate of cell division appears to vary to an extent which is not expected from the pattern of fibre length growth on the midside (Figure 5.3). This is opposed to the suggestion put forward in the introduction for this experiment, that cell division is relatively constant whilst keratinisation varies.

The estimated rate of cell division and fluctuations observed in the present experiment were of a similar order of magnitude to the fluctuations previously demonstrated in a Lincoln ram (Scobie and Hynd, 1987). The highest observed **median** mitotic rate for skin samples collected during the present experiment from ewe L1 was 7.36 cells per average follicle bulb section during two hours of arrest with colchicine, which was 35 % greater than the lowest recording of 5.44. During the previous experiment, the Lincoln ram exhibited a maximum **median** mitotic rate of 14.6 cells per average follicle bulb section during three hours of arrest with colchicine, which was 55 % greater than the lowest recording of 9.4. When compared on a per unit time basis, these values were slightly lower for the ewe (3.68/hr. max., 2.72/hr. min.) than for the ram (4.86/hr. max., 3.13/hr. min.).

Fluctuation in cell division rates between sites on the midside of ewes L2 and L3 do not correspond with those we would expect based on the differences in fibre length growth rate of ewe L1. For example the 0200 and the 0400 samples were collected from neighbouring sites and exhibited a difference of 2.73 and 1.91 cells arrested over the two hour period for ewes L2 and L3 respectively. The sites therefore showed more than a 20% difference in cell division rate, although they lay very close together on the midside (100 mm apart), in a region which was relatively uniform in wool growth rate, based on the length growth rate observations from ewe L1.

Since the skin biopsies came from separate animals to the autoradiography measurements, there may be differences in the sites between the sheep, as there seems to between the sites on an individual. Hynd *et al.* (1986) had previously shown that variations between midside sites on a Merino wether were negligible, but that samples taken from the back exhibited a lower rate of cell division than samples taken from the side. Given the magnitude of the differences in wool growth across the side of the animal in the present study, the results of ^{35}S autoradiography presented here conflict with those of Hynd *et al.* (1986). Statistical analysis of the present experiments were therefore necessarily rudimentary, and it is suggested that in the future, experiments in breeds other than the Merino should be designed with a covariate analysis in mind. All experiments which were not directed at examining circadian patterns of cell division, but used a pattern spaced across the midside were conducted on Merino wethers. Merinos showed the smallest circadian fluctuations in Chapter Two, and based on evidence of the Hynd *et al.* (1986), little variation across the midside. Fluctuations in wool growth rate are therefore not considered to have influenced the results of such experiments in the present thesis.

There was some indication that cell division rates fluctuated during the early hours of the morning (Table 5.1). It is interesting to note that this has been the case in all of the experiments conducted in this laboratory which have been designed to study the circadian pattern of cell division rates. That is, in the morning, around the time the lights were switched on and/or the animals were accustomed to being fed, there has consistently been some deviation in the rate of cell division and most commonly a reduction. Some aspects of these experiments are examined below in a search for the common characteristics of all, (the related work published by Hynd *et al.* (1986) has been excluded from this revision, as it was drawn from a Ph.D. thesis (Schlink, 1977) and the specific details were not apparent).

Lights were switched on at 0600 hours in the present experiment, compared to 0700 hours in both the first experiment in this thesis (Chapter Two), and the experiments of Scobie and Hynd (1987). Lights were switched off at 1800 hours in the present experiment compared with 2015 hours in the experiments of Scobie and Hynd (1987) and 1715 hours in the experiment of Chapter Two. The objective of these regimen was to maintain daylength conditions in all of the experiments similar to those experienced by the animals immediately prior to the time they were

moved into the room. Changing lighting patterns has previously demonstrated that seasonal wool growth rates are slow to change (Slee, 1965), and it was considered most likely that a similar effect would occur in these experiments.

The length of daylight in the present experiment (12 hours) was shorter than that experienced by the animals in the experiments of Scobie and Hynd (1987) (13 25 hours) and longer than in the first experiment of this thesis (10.25 hours). This intermediate daylength was considered unlikely to have biased the results of the work described in this chapter.

Feeding/cleaning commenced at 0900 hours in the present experiment and also in those of Scobie and Hynd (1987), whereas feeding times were 0800 to 1000 and 2000 to 2200 hours in the experiments of Chapter Two. Thus feeding/cleaning was relatively close to lights on for the morning fed group in the experiment of Chapter One, but nearly three hours after lights out for the evening fed group. For the animals used in the experiments of Scobie and Hynd (1987), feeding/cleaning was only two hours after lights on, whereas those in the present experiment were fed three hours after lighting commenced.

It is possible that stimuli or timing signals throughout the day in the present experiment did not coincide as they may have in previous experiments (Chapter Two) and Scobie and Hynd (1987). That is the sheep in the experiments of Scobie and Hynd (1987) and Chapter Two could have become accustomed to expecting food shortly after the lights came on and as a result, become more excited than the animals used in the present experiment. In the light of the findings of Chapter Four, excitement and feeding which cause the release of catecholamines (Tindal *et al.*, 1985) could have produced the results observed in Chapter One.

A final point of interest is that of temperature.fluctuations within the controlled environment rooms during each experiment. This was monitored using a maximum/minimum mercury thermometer for the duration of each of the experiments. During the experiments of Scobie and Hynd (1987) the temperature ranged between 18 and 26 °C. A much older and poorer light room was used for the first experiment of this thesis, the door of which was often opened during the day to supplement the poor ventilation and air conditioning. The temperature varied

between 13 and 26°C (Ave. min. 15.5, Ave max.18.7) and was governed by the temperature of the external environment to a large degree. It was evident that the changes followed the diurnal increase and decrease of the external environment, however this data was not specifically recorded. For the experiment of the present chapter, a new air conditioning unit was installed in a more appropriate controlled environment room for the experiment. The temperature was maintained at a higher and more even level for the duration of the experiment, an average of 23°C (± 2) was maintained, despite outdoor temperatures of up to 40°C and as low as 14°C.

Noradrenaline release in response to cold was demonstrated to have potent effects on cell division in Chapter Four, and it is possible that this may have influenced cell division rates observed in the experiment described in Chapter Two. Temperature was not perceived as a major contributing factor at the commencement of the early work, and the potential effects of skin cooling only became evident as the experiments of Chapter Four progressed. The variation in temperature during the present experiment was minimal under the circumstances and relatively warm compared to the temperatures required to elicit a change in cell division during Experiment 4d. Naturally, further work should be attempted using imposed diurnal temperature rhythms to test these ideas, but the outcome of such investigations can be predicted with some confidence.

In conclusion, the experiments of this chapter provide further evidence to suggest that the mitotic activity of wool follicles does not exhibit a smoothly fluctuating circadian rhythm. The fact that variation in length growth rate occurs across the midside of animal L1 was an important observation of the present investigation, as differences in cell division rate in midside sites was not apparent in a Merino studied by Hynd *et al.* (1986). High rates of wool growth, and therefore cell division in the wool follicle are more likely to yield significant differences between sites, a major reason for the choice of breed during the present experiment. However, the fastest and slowest sites for wool growth rate did not coincide with the sites that exhibited the extreme differences in cell division, which were observed at neighbouring sites within a region that showed the greatest rate of wool growth. The fluctuations in cell division rate that have been observed in sequential samples across the midside, are therefore not fully explained by the variation in fibre growth rate determined in the immediately surrounding skin (of a

similar animal) and are considered likely to be a consequence of circadian changes in the level of catecholamines. It is suggested that an experiment which utilises the rate of wool growth of a given site as a covariate, be conducted to further examine and confirm the erratic fluctuations in cell division rate which have been implicated by this thesis.

Chapter Six.

General Conclusions.

" 'How long ?' asked Durf again.

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

6.1. Summary of the present work.

The technique of using a small intradermal dose of colchicine for the measurement of cell division rate in wool follicles has been refined and used throughout this thesis. This work is collated in Appendix One and Two. Briefly, colchicine arrests dividing cells at metaphase in the skin of the sheep by binding to microtubules, and since this binding occurs in a stoichiometric relationship, it is necessary to establish an optimum dose for each new breed of sheep studied. A frequency distribution of the number of arrested cells per random follicle bulb is obtained using this method, and essentially, the higher the average number of arrested cells per bulb, the faster the rate of wool growth. Since the frequency distributions obtained are not normally distributed, the statistical methods used to analyse the data and the reasons for the choice of analysis are outlined in Appendix Two.

Earlier work implied that a sinusoidal circadian rhythm was evident in cell division rate in wool follicles. Ideally, basal cell division rate should not fluctuate if the measurement of cell division in the wool follicle is to be used to indicate likely changes in wool growth rate. The two separate investigations carried out during the course of experiments reported in this thesis, failed to find any supporting evidence for this sinusoidal rhythm, however, erratic fluctuations in the rate of cell division were detected. Given the discrepancy between the findings of the present investigations and other experiments, it is considered possible that the basal rate of cell division was actually being influenced by the experimental protocol designed to measure it. The influence of the catecholamines implicates their involvement in these fluctuations, whereas the long response time required for cortisol levels to influence cell division precludes it.

Elevated levels of plasma cortisol will reduce cell division rates in wool follicles, but the time taken for the response to become apparent is of the order of one day. In contrast, the catecholamines proved to have profound effects on cell division rate in wool follicles within periods of a few hours, both *in vivo* and *in vitro*. Local injections of a chemical sympathectomising agent (6-hydroxy-dopamine) were shown to promote cell division relative to control sites in some preliminary experiments, yet not others. Portions of the sympathetic nervous system were removed by surgical means, and the major source of noradrenaline

therefore eliminated from a small region. When exposed to a cold environment, cell division rate was reduced in biopsies removed from non-sympathectomised sites, which suggest that physiological levels of noradrenaline are adequate to affect cell division and therefore wool growth.

6.2. Guide-lines for future research.

The arsenal of methods now available should permit us to establish a greater understanding of factors which can influence the events that lead to the production of a wool fibre. However, the following precautions should be taken.

The amount of stress which is likely to be created by the circumstances should always be kept in mind, and attempts should be made to minimise excitement. It is important that animals be afforded adequate time to become familiar with their surroundings and handling procedures since confinement has been shown to increase cortisol levels for several days (McNatty *et al.*, 1972), which may be adequate to bring about some changes in wool production based on the observations of Chapter Three.

The most profound fluctuations in cell division rate occurred in the morning hours in the experiments of Chapter One and Chapter Five, and biopsy samples were removed during the afternoon in other experiments for this reason. It is considered advisable that this practice be continued in similar work with sheep maintained indoors and accustomed to morning feeding and cleaning procedures. The sinusoidal circadian rhythm in depilation force observed by Foldes *et al.* (1985) may also involve the catecholamines, and this may warrant a detailed investigation.

The implications of these observations with respect to experiments conducted on animals maintained outdoors are unclear, and should be investigated in the future. Food intake and activity patterns would probably differ from those observed under pen feeding, and may have an important bearing on the erratic circadian fluctuations in the basal rate of cell division.

Similarly, the excitement involved with rounding up, yarding and handling animals may lead to aberrant observations.

With respect to commercial farming of sheep for wool, infrequent excitement induced stress and the subsequent release of adrenaline is unlikely to influence annual wool production. Short term changes in the level of plasma cortisol are similarly unlikely to reduce wool production, however when elevated levels are maintained for periods longer than twenty-four hours the reduction in wool production per annum may become important. In contrast, frequent and brief periods of cold environmental temperature are likely to cause a reduction in the amount of wool produced through the release of noradrenaline and its sequelae, this will be of greatest significance in poorly-fleeced animals and on the extremities of well fleeced animals. It is important to appreciate that these predictions will also depend on the extent to which other hormonal, metabolic and behavioural compensation can be made in the longer term (e.g. elevated food intake or acclimatisation), all of which are factors requiring some attention.

Temperature is also of real concern to experiments which set out to examine changes over time using the colchicine technique, and endeavours to control and monitor temperature should be made. The results of Cunningham *et al.* (1979) and the *in vitro* results presented here suggest that catecholamines exert an influence independent of blood flow. Since peripheral vasoconstriction is a major effect of both noradrenaline and adrenaline, the presence of either will reduce blood flow and therefore temperature. Likewise, reduced environmental temperature will cause the release of noradrenaline and thereby cause vasoconstriction and reduced blood flow. It is therefore difficult to separate these effects *in vivo*, but the effects of blood flow to the skin, on wool growth must be addressed in the future. However, the results of *in vitro* analysis suggest that some components of wool production may respond to catecholamines independently of their effects on blood flow. It is also considered very important to keep in mind the site of release of the catecholamines; nerve terminal release of noradrenaline in the skin will place the neurohormone in the vicinity of the follicle, whereas release from the adrenal gland will cause vasoconstriction which will preclude the hormone from reaching the follicle.

The bulk of evidence presented is thus of great significance to the design of experiments, and adequate controls and settling in periods should be catered for. The use of a controlled environment room is advocated for detailed studies. Where possible, intensive sampling regimen should be spread out as in Chapter Five, rather than concentrated as in Chapter Two, in an effort to minimise the cumulative effects of repeated sampling and handling distress.

6.3. Hypothesis for the control of wool growth.

To quote Hutchinson (1965): "Thus, the possibility still remains that the rhythms of shedding and wool growth are two separate rhythms. However this may be, it is an interesting fact of evolution that man, by breeding, has apparently changed the cycle of only the part of the coat that is valuable to him, whereas the rest still continues the original cycle.". The present author concurs with this hypothesis, and would like to add that cell division in these wool growing areas is controlled by mechanisms akin to those which control cell division in the epidermis. It would appear that the major wool growing portions of the skin of the sheep have become less responsive to controls which regulate the growth of the hair. The work of Bullough (1962, 1965) suggests that division in the epidermis is controlled by the catecholamines, yet that of the hair follicle is not. The evidence presented in Chapter Four shows that many aspects of the wool growth are influenced by catecholamines, in contrast to the hair follicle. [N.B. Although the Experiment 4d was conducted on the skin of the cheek, all the animals were chosen for a high level of face cover. The biopsy site was low on the cheek, well inside the boundary of the wool-growing area and therefore essentially a sample of wool-growing skin. In support of this, the average rate of cell division was similar to that recorded in biopsies collected from the midside.] A recent review by Brown (1991) presents numerous accounts of circadian rhythms in cell proliferation in mouse, rat and human epidermis, and although only erratic fluctuations have been observed in the wool follicle, they may share many regulatory agents in common.

6.4. Sheep and the growth of wool: Keeping them in perspective.

As a concluding remark, it is very important for us to keep in mind the fact that a sheep is an animal first and foremost, and the growth of wool is a by-product of survival as far as the

sheep is concerned. Figure 6.1 has been adapted from Morrison (1966) and diagrammatically shows the length of "fur" on the body regions of a Guanaco (*Lama guanicoe*). The guanaco lives in South America and its range extends from sea level to 4500 metres altitude, in a region of large contrasts in temperature from the extremely dry and hot Atacama Desert to sub-zero temperatures with wind in Tierra del Fuego. This range of temperatures is not so very different from those imposed on sheep in the regions man chooses to keep them. The ability to dissipate or conserve heat under such conditions is critical for the survival of the Guanaco. The very short fur of the extremities and axilla (Figure 6.1), allows the animal to exchange heat with the environment from these "thermal windows", utilising both vasoconstriction and posture.

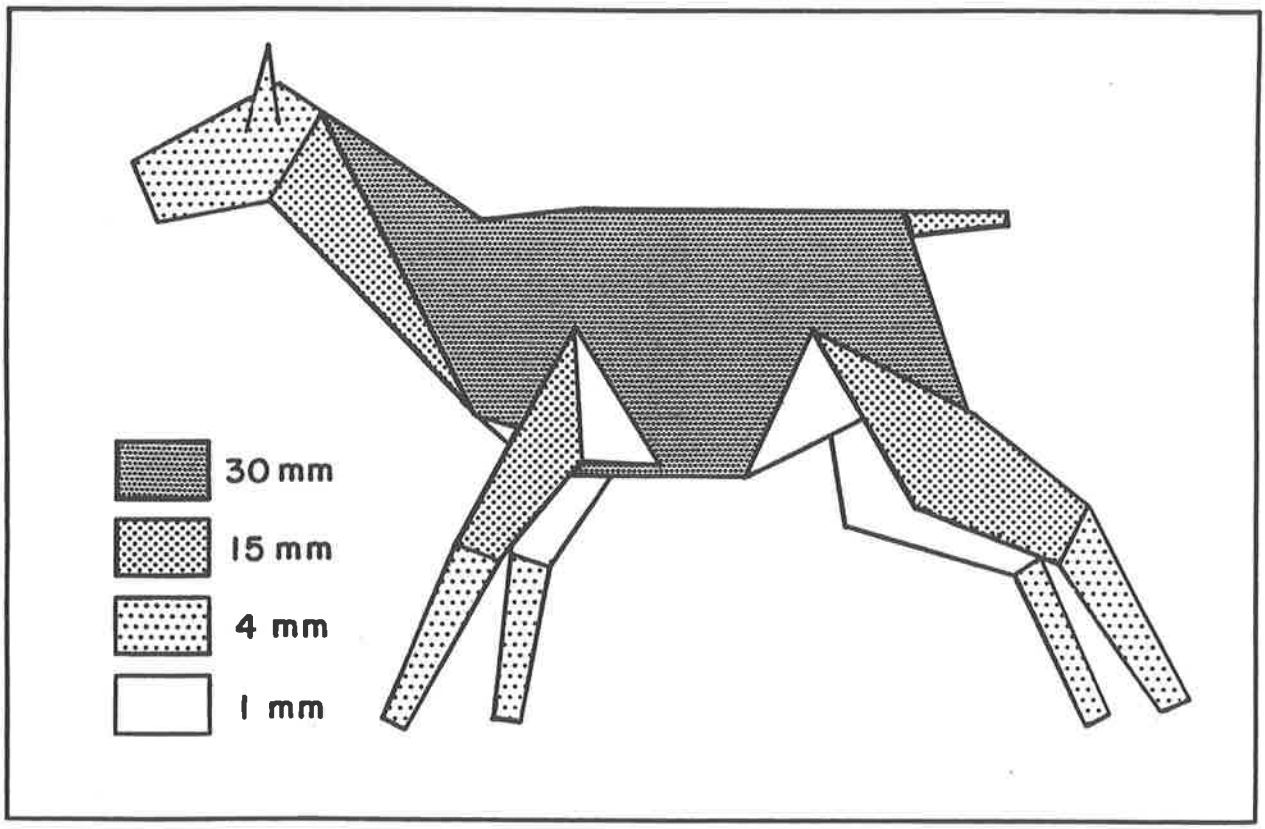


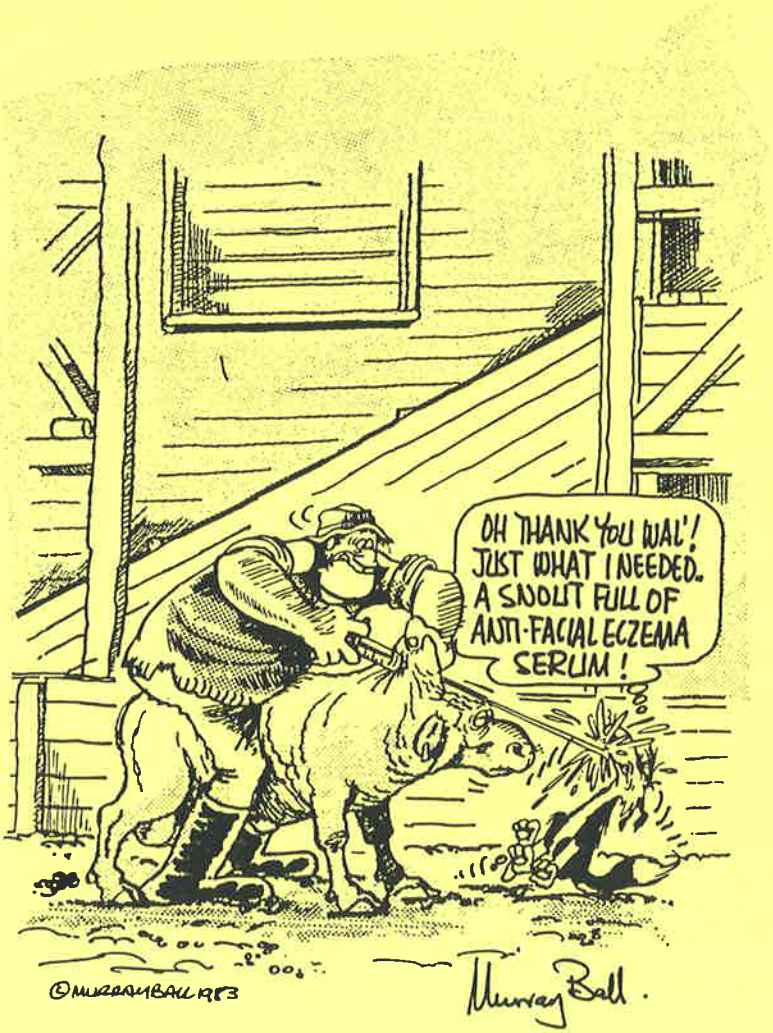
Figure 6.1. Diagrammatic representation of the length of the coat of the Guanaco. Adapted from Morrison (1966).

With slightly shorter legs, more wool over the head and an adjustment of the lengths of the fleece, Figure 6.1 could satisfactorily represent a generalised breed of sheep. If we maintained a region of short fleece on the midside of this sheep by close-clipping a patch, we would

provide another "thermal window". It is therefore not surprising that, Wodzicka-Tomaszewska and Bigham (1968) observed differences in the rate of wool growth depending on whether the patch was insulated or not. Based on the evidence presented in Chapter Four, release of noradrenaline under the influence of cold would be expected to slow the rate of wool growth on uninsulated patches. Complete shearing of the animal would eliminate this type of control, which could explain the discrepancies between the studies of Wodzicka-Tomaszewska and Bigham (1968) and Bigham (1974). After shearing, the animal must compensate by changes in food intake and metabolism and many of the factors that affect wool growth would change together. Many a farmer has found to his misfortune (and more so to the misfortune of his flock), that such a metabolic compensation cannot be made quickly, and large losses caused by hypothermia may occur after shearing. Various techniques of shearing used to overcome this problem, aim to leave a greater length of fleece on the body.

Appendix One.

The Intradermal colchicine technique.



Taken from Ball (1985).

A1.1. Introduction.

Colchicine is a metaphase arrest drug belonging to the vinca alkaloids and has been used in the study of cell kinetics under a number of conditions, including application to the study of wool follicle cell kinetics (Schinckel, 1961, 1962; Fraser, 1965; Short *et al.*, 1965; Wilson and Short, 1979; Hynd *et al.*, 1986; Williams and Winston, 1987) The mechanism by which colchicine works is to bind to tubulin polymers and prevent them from assimilating to form the microfibrils which separate chromosomes at metaphase of cell division (Borisy and Taylor, 1967a and b). This process is reversible and since the binding of colchicine to tubulin is a stoichiometric phenomenon, then an under-supply of colchicine to the cell system of interest results in a number of mitoses escaping arrest. Bertalanffy (1964) showed that both colchicine and tritiated thymidine yielded similar estimates of cell turnover time in various tissues, if samples treated with colchicine were taken during a period of linear accumulation of metaphase arrested cells. This led Tannock (1965) to suggest that a dose response curve and linearity of arrest should be determined for each and every system to be studied.

Hynd *et al.* (1986) modified the technique of Schinckel (1961, 1962) to produce a method of applying colchicine-induced metaphase arrest to the study of cell division rates in wool follicles. Dose response curves and linearity of arrest were established in these studies. Throughout the course of the present work and in other studies (P.I. Hynd personal communication) it became apparent that the original formula for successful arrest was inadequate in some situations. As a result the original method was further modified and these alterations are reported in this appendix.

For each new sheep skin system or breed studied throughout the course of the present experiments, it was necessary to establish an appropriate dose rate adequate to produce complete arrest. In all cases it was assumed that the appropriate dose determined in a two hour *in vivo* dose rate trial would give linearity of arrest over a period of two hours. This assumption must be justified *a priori*, and the following outlines the major reason for adopting this approach. The bulk of the experiments described in this thesis were designed primarily for the study of the effects of the stress hormones on wool growth. Since frequent sampling

associated with a linearity trial could impose undue stress on the animal, it was considered that this might ultimately bias the result of such an experiment.

In the light of earlier work (Hynd *et al.*, 1986; Scobie and Hynd, 1987), it was considered possible that under some circumstances circadian periodicity of cell division rates in wool follicles may exist. A change in the basal rate of cell division over the period of colchicine arrest would naturally have lead to a change in the rate of accumulation of arrested mitoses, and therefore, a lack of linearity of arrest. Although the experiments described in Chapters Two and Five are at odds with earlier findings (Hynd *et al.*, 1986; Scobie and Hynd, 1987), they also suggest that the rate of mitosis is dynamic and not constant.

The same lack of linearity could apply to the influence of a hormone over time. That is, irrespective of whether a hormone has a stimulatory or inhibitory effect, we would expect any influence to become evident over a certain time period. Colchicine administered at the time of hormone challenge would therefore cause mitoses to accumulate over a period of changing rates of cell division, which would present a different environment to that used to examine linearity of arrest. (It must be remembered when comparing plasma hormone levels with cell division rates, that the former is a value apparent at the time of sampling and the latter is the summation of all mitoses arrested over the time between the injection of colchicine and the biopsy.) It was for these reasons that a contemporaneous control sample was taken (where possible) and used for comparison in preference to treatment and control samples taken at different times. Where this proved impossible, biopsies were usually collected at the same time of day, when taken on consecutive days or at infrequent or weekly intervals. This system was adopted as the standard means of assessing the action of various treatments on cell division throughout this thesis.

A further modification to the original method (Hynd *et al.*, 1986) adopted during the course of the experiments reported in this thesis was the discovery that the use of a smaller vehicle volume and correspondingly higher drug concentration to supply an equivalent dose resulted in a more reliable arrest of mitosis (Scobie and Hynd unpublished observations). Thus the relevant dose of colchicine was delivered in 0.1 ml of saline (0.9 % (w/v) NaCl) rather than 0.2 or 0.3 ml as in some of the experiments of Hynd *et al.* (1986) (Experiment A(i) of this

appendix, and the experiments described in Chapter Two of this thesis were undertaken using 0.2 ml of saline). Presumably the smaller amount of saline disrupts normal cellular events to a lesser degree and/or creates a higher tissue concentration of the drug and/or a slower rate of removal of the colchicine by the circulation.

Finally the drug has a toxic effect, not only on whole animals (Short *et al.* 1965, Wilson and Short 1979), but also on cells, in terms of the rate of DNA synthesis (Hell and Cox, 1963; Fitzgerald and Brehaut, 1970). Some of the observed toxic effects of the drug on cells of the wool follicle are described in this appendix. Hence, shorter periods of arrest (two hours) were generally applied throughout the experiments reported in this thesis.

In summary, the effects of colchicine vary under different conditions and this must be taken into account when comparing measurements made using this drug. As a result, the mitotic arrest method originally described by Hynd *et al.* (1986) evolved along with the course of experiments reported in this thesis (and elsewhere). Some of the events of this gradual development are reported here as an appendix, to prevent repetition and allow the rest of the text to flow in an unhindered manner. Where relevant, reference to this appendix is made within the report. In cases where the observation was immediately important to the prevailing discussion it has been included at that point for clarity.

A1.2. Experiment A(i).

Aim: To determine an optimum dose rate of colchicine injected into the dermis to achieve mitotic arrest in the skin of Tukidale, Corriedale and Merino wethers under a given feeding and lighting regime.

A1.2.1. Experimental Procedure.

Three two tooth wethers (1 Tukidale, 1 Corriedale and 1 Merino) were housed indoors, and fed a ration as described in Chapter Two (Section 2.8(b)). After four weeks of feeding this ration, samples were taken from these animals according to the pattern shown in Figure A1.1.

Colchicine (A.R. grade, Sigma Chemical Co., St. Louis, Missouri, U.S.A.) at levels of 0, 0.5, 1, 5, 10, 50 and 100 µg dissolved in sterile physiological saline (0.2 ml) was injected into the dermis with a 27 gauge hypodermic needle. The total volume of solution delivered was 0.2 ml. Skin biopsies were taken 2 hours later from the site of injection and treated using the haematoxylin and eosin method of Hynd *et al.* (1986).

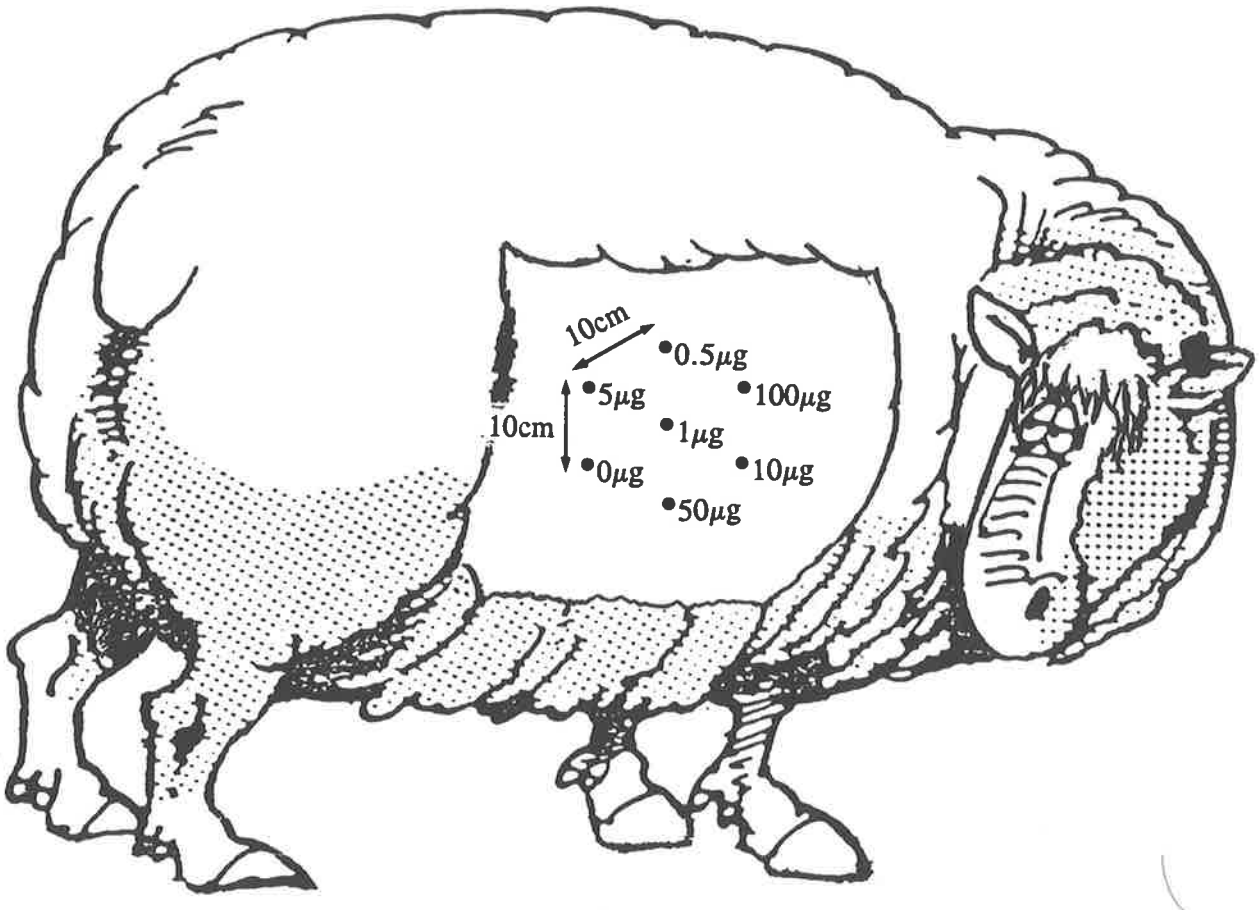


Figure 1.1. The site and dose of intradermal injections of colchicine and subsequent biopsy samples collected from Merino, Corriedale and Tukidale wethers.

A1.2.2. Results and Discussion.

The results are depicted in Figure A1.2. From this graph, the most suitable doses of colchicine should be those which achieved the greatest arrest of cell division. These were as follows: Tukidale, 0.5 µg; Corriedale 1 µg; Merino 0.5 µg. However, these dose rates were far lower than those determined by Hynd *et al.* (1986), and thus questioned since this was contrary to expectations. Upon closer examination it became apparent that counting errors had arisen from the fact that some cells had continued to divide in the presence of colchicine as shown in Plate

A1.1. To eliminate this type of counting error, a subjective criterion was established such that mitotic figures of this type were searched for in each of the samples. The samples were re-examined by this method, and a dose rate deemed acceptable if a rapid search of roughly one hundred random bulb sections revealed no such figures. At the time of this experiment, this was deemed to be a more acceptable index than the dose corresponding to the highest cell division rate. The artificially high arrest rates assessed in this manner have been indicated on Figure A1.2, (*) and the dose rates subsequently chosen were as follows: Tukidale 50 µg; Corriedale 50 µg; Merino 10 µg.

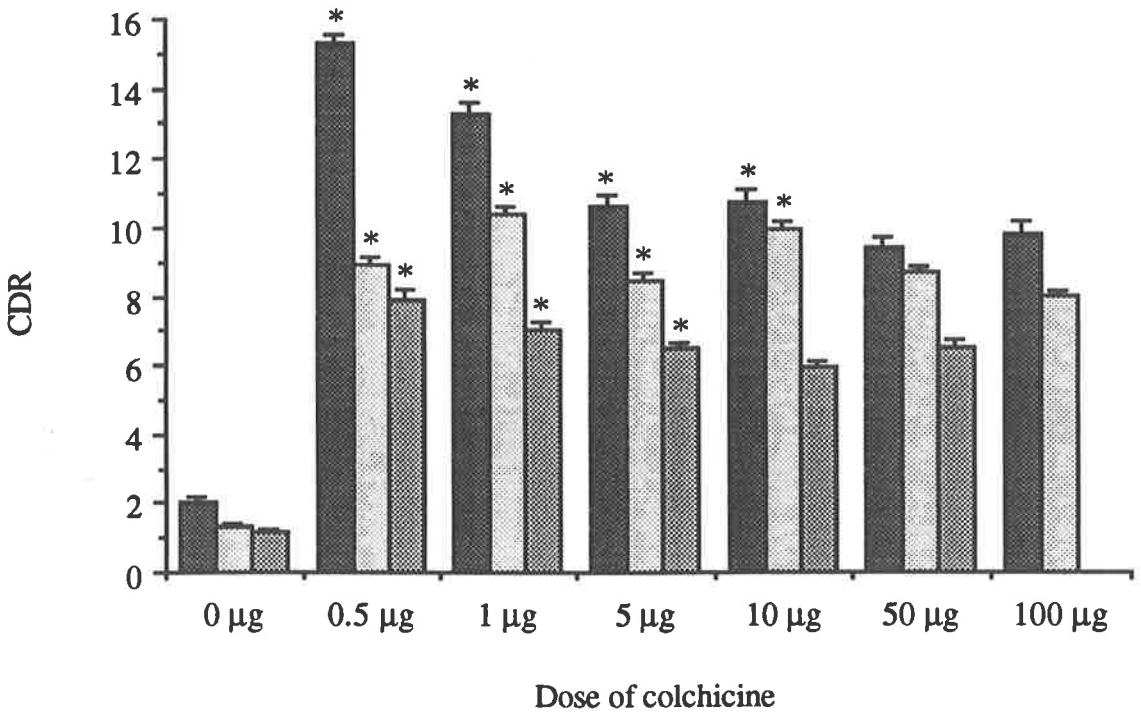


Figure A1.2. The number of arrested mitoses at various doses of colchicine injected into the dermis of Tukidale ■, Corriedale ▩ and Merino ▩ wethers.

It is interesting that this phenomenon was also apparent in the merino sheep in the present experiment but not in the animals used by Hynd *et al.* (1986). Since Hynd *et al.* (1986) took samples three hours after colchicine injection, and only a two hour period of arrest was imposed in the present experiment, it is possible that the pattern of arrest could have been a result of the shorter time the cells were exposed to the drug.

The breed difference in the effectiveness of the drug was probably a consequence of the thicker skin of the two breeds with faster growing fleeces (Corriedale and Tukidale). Thicker skin could provide a greater intradermal space for the drug to disperse into and/or a higher rate of removal due to increased vascularity. These factors could lower the 'effective dose' at the level of the bulb. In support of the latter reason, it was noted that the 0.2 ml of saline, although injected in a similar manner in all animals, left a much larger and longer lived bleb on the skin of a Merino than either of the other two breeds.

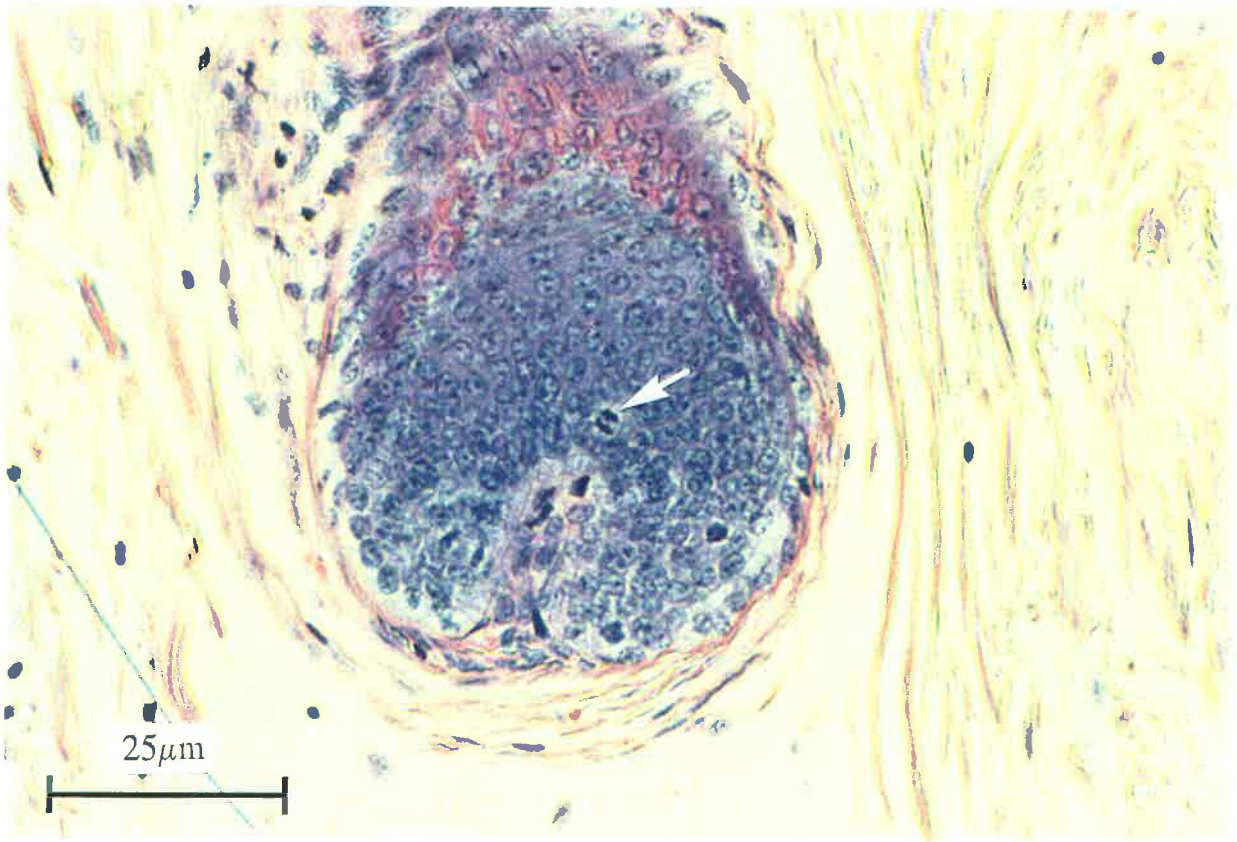


Plate A1.1. A random follicle bulb in which it is apparent that the colchicine treatment has not completely arrested cell division. The arrow indicates a cell which has continued beyond metaphase.

A1.3. Experiment A(ii).

The following experiment was designed to determine the optimum intradermal colchicine dose rate to achieve mitotic arrest in Lincoln ewes.

A1.3.1. Experimental Procedure.

Six Lincoln ewes were housed in a controlled environment room under the conditions imposed in Experiment 5a and 5b. After six weeks the ewes were prepared for the removal of colchicine samples and doses of 50 and 100 μg (in 0.1 ml of saline) were administered into sites lying directly over the tenth rib (50 μg uppermost and separated from the 100 μg site by 10 cm). The biopsies thus obtained were processed by the method of Hynd *et al.* (1986), assessed for mitotic figures such as those shown in Plate A1.1, and then counted as per the method of Schinckel (1962).

A1.3.2. Results.

The results are shown in Table A1.1 and it was noted that samples taken from sheep L3 and L6 exhibited a number of split mitoses, as in Plate A1.1. In all animals the rate of cell division was smaller after treatment with the 50 μg dose, therefore the dose rate of 100 μg was chosen for use in Experiment 5b since it produced a more reliable arrest of mitosis in these animals.

Table A1.1. The average number of mitoses arrested by two doses of colchicine (50μg and 100μg) in skin samples collected from six Lincoln ewes (L1 to L6).

	Mean CDR (± s.e.m.)	Mean CDR (± s.e.m.)	
Sheep Number	50 μg dose	100 μg dose	Comments
Sheep L1	9.51 ± 0.24	9.64 ± 0.26	
Sheep L2	9.84 ± 0.40	13.69 ± 0.25[a]	
Sheep L3	6.37 ± 0.29	9.93 ± 0.37[b]	Split mitoses at 50 μg
Sheep L4	11.77 ± 0.31	12.19 ± 0.25	
Sheep L5	10.98 ± 0.28	10.99 ± 0.26	
Sheep L6	8.30 ± 0.25	9.23 ± 0.21[c]	Split mitoses at 50 μg
Mean (± s.d.)	**9.46 ± 1.93**	**10.95 ± 1.72**	

[a,b,c] Significantly different to the 50 μg sample using the Kolmogorov-Smirnov test.

A1.4. Experiment A(iii).

Aim: To investigate whether the toxic effects of a small intradermal dose of colchicine have a lasting effect on wool follicle cell division rates.

A1.4.1. Experimental Procedure.

The animals described in Experiment A(iii) were treated with similar doses of colchicine (100 μg) on sites neighbouring the position of those outlined in that experiment. Biopsies were not removed on this occasion, and the sites were clearly marked. After three days, the same injection protocol was repeated and on this occasion 1 cm biopsies were taken two hours later under local anaesthetic and treated as in Experiment A(ii).

A1.4.2. Results.

Pretreatment of sites with colchicine consistently yielded samples that showed signs of perturbed cell division. Indeed, it proved impossible to obtain measurements of cell division by the method of Hynd *et al.* (1986). The Plate A1.2 is a photomicrograph of a histological section of a biopsy obtained from ewe L3 which shows a lack of arrested mitoses, and follicles that appear to have degenerated. Plate A1.3 is a photomicrograph of a histological section of a biopsy from the same animal obtained during the experiment described in the previous section (100 μg dose). This plate shows normal mitotic arrest, and follicles which resemble those observed in all other animals studied during the experiments described in this thesis. Clearly, long-term treatment with colchicine will perturb cell division.

A1.5. Discussion.

The vinca alkaloids have been used as chemotherapeutic agents in the treatment of cancer (and to promote polyploidogenesis in plants) for the same reason as they have been used here. These drugs have the capacity to reversibly inhibit cell division and subsequently be removed by metabolism and excretion (or washing), which makes it possible to selectively kill a rapidly

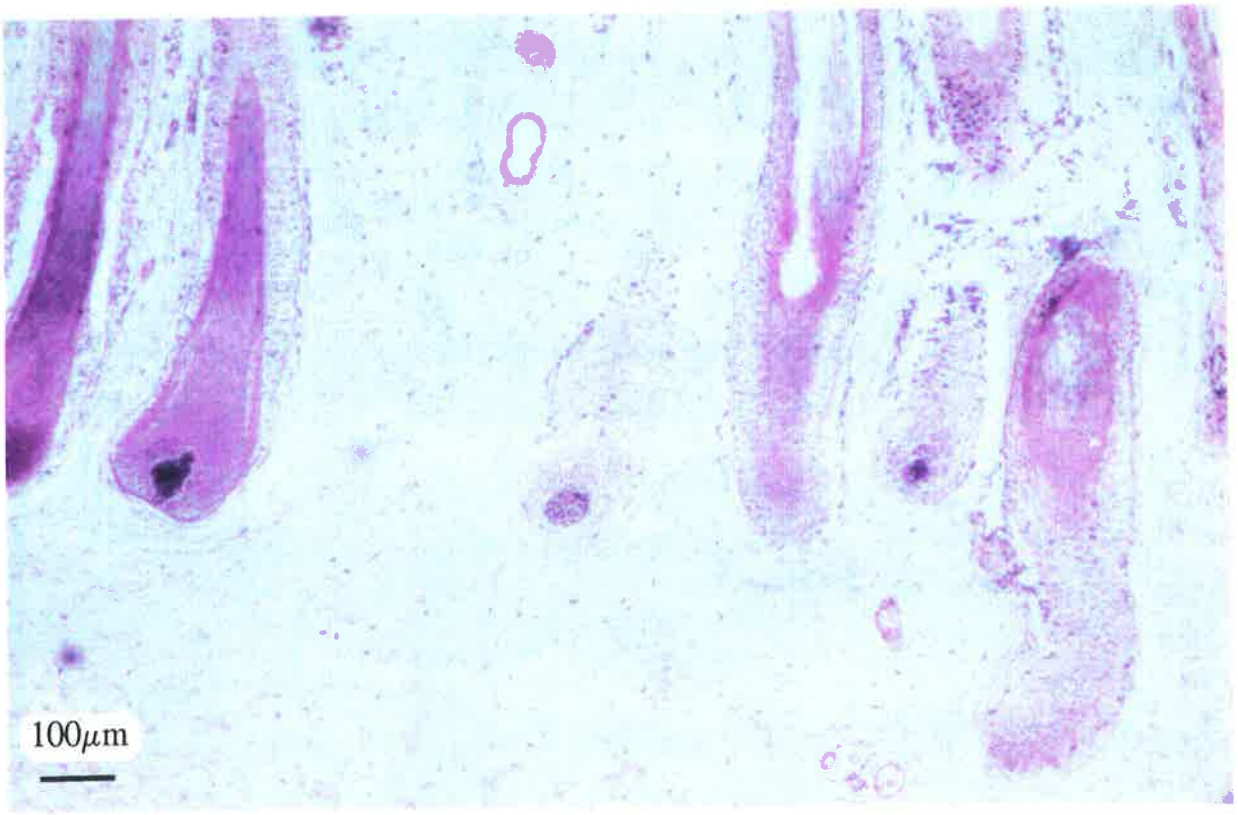


Plate A1.2. Photomicrograph of a histological section of the skin of Lincoln ewe L3, showing signs of follicle degeneration after treatment with colchicine.

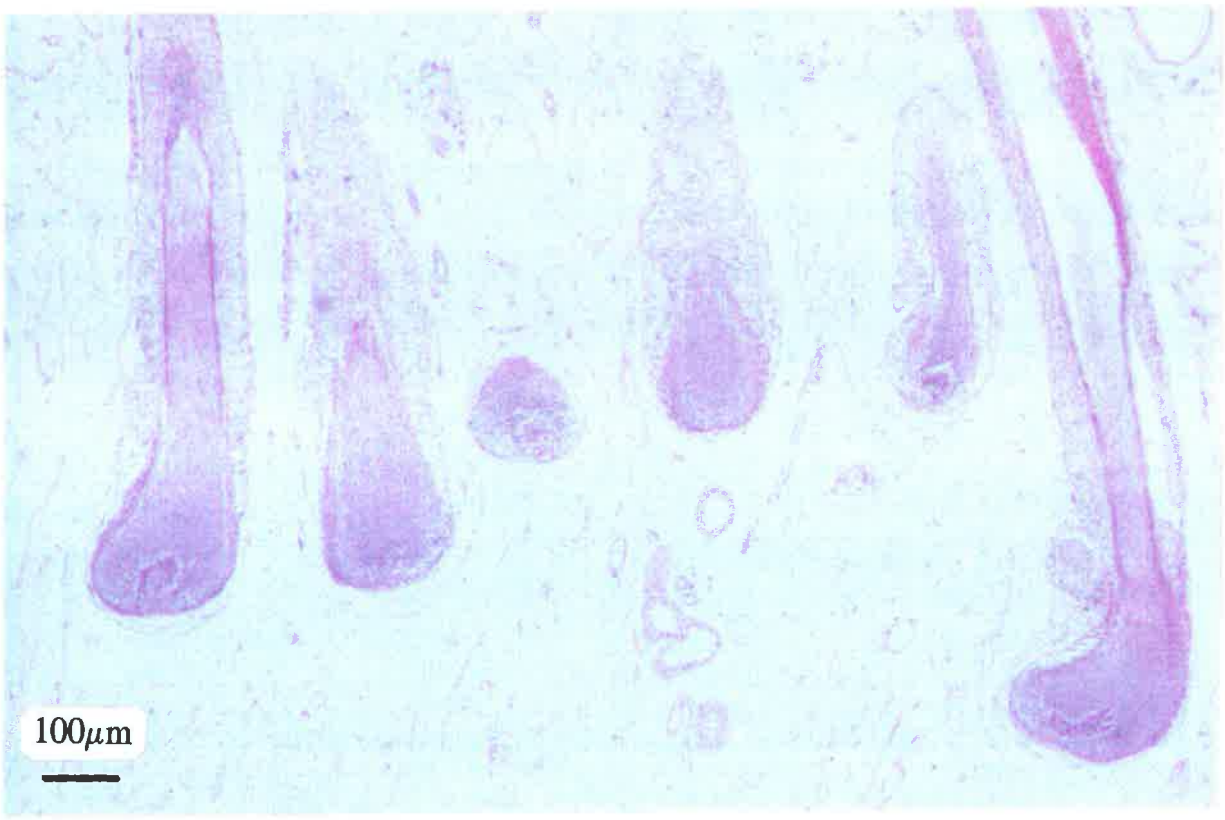


Plate A1.3. Photomicrograph of a histological section of normal skin of Lincoln ewe L3. Normal metaphase arrest is apparent in the follicle bulbs.

dividing cell line such as a tumor and leave others relatively unharmed. Chemotherapy may result in the loss of hair of the patient, and although fibre loss was not observed in the present experiments, evidence for the cessation of wool follicle cell division has been obtained.

For this reason, special care must be taken to ensure that sample sites are not placed closer than at least four centimetres from a previous site. Hynd *et al.* (1986) did not observe any appreciable arrest of mitotic figures at a distance of five centimetres from the site of injection and four to five centimetres may therefore be considered the lower limit of distance between samples. In the light of comments made in Section A1.1., this may be attributable to a difference in the dispersion of the solution into the tissue. Since the 0.2 ml of saline left virtually no bleb at all in these animals, it was subsequently decided to inject the colchicine into a previously marked site rather than post-injection marking the site as in the method of Hynd *et al.* (1986).

Further evidence for the toxic effect of colchicine is shown in Plate A1.4 which depicts an animal that was used in Experiment 4d. There was an obvious shedding of fibres from the area surrounding the sample site. This was only the second occasion such shedding has been observed in this laboratory, despite the large number of animals examined. Comparable samples taken from the midside of this breed of sheep failed to produce such shedding on any occasion (N.B. Over 300 such midside samples from Merino sheep used in experiments for this thesis alone).

The other observation of this type of fibre shedding was made following colchicine sampling of the Suffolk ram during the experiments of Scobie and Hynd (1987). After sampling this animal demonstrated a cessation of fibre growth followed by the growth of black fibres from a region of about two to three centimetres in diameter surrounding the sample site (unpublished observations). Since the bulk of DNA synthesis (Downes and Wilson, 1971; Ward and Harris, 1976) and cell division occurs within the follicle, the lower density of follicles and thin skin of this breed could be expected to increase the dispersion of the drug, and also the effective dose per follicle and thereby produce the observed effect.

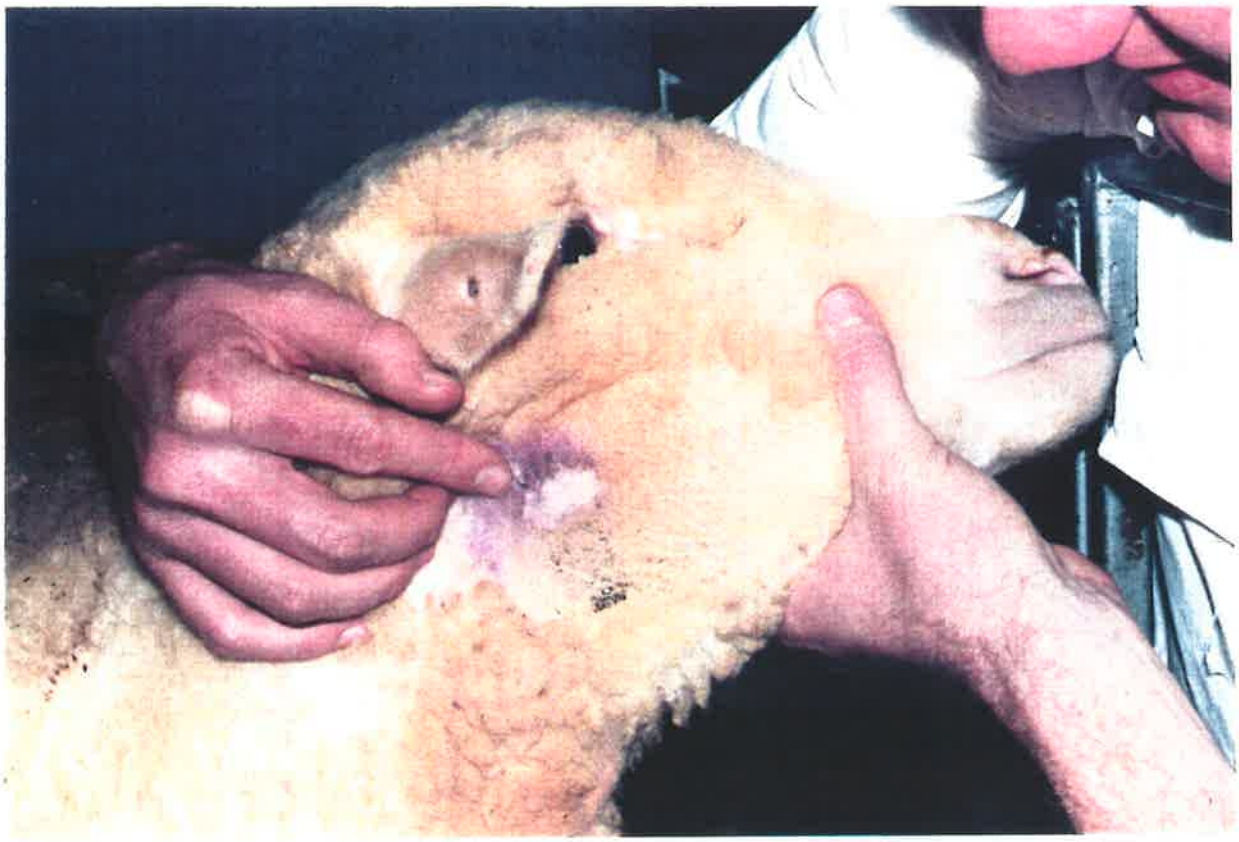


Plate A1.4. New fibre growth at the site of the intradermal colchicine injection. This indicates cessation of growth, followed by recovery and shedding of the original wool fibres.

Corriedale, Tukidale and Lincoln sheep used in other experiments reported in this thesis failed to demonstrate obvious shedding around a site from which a skin sample had previously been removed. The Lincoln ewes in Experiment A(iii) demonstrated a cessation of cell division, however during follow up observations, shedding over the site was not evident. It is possible that the biopsy of skin followed by profuse bleeding removes a large percentage of the colchicine administered, and in this manner, the cessation of growth may not be as marked as demonstrated in a sample bathed in colchicine for a prolonged period as was the case in the Lincoln ewe (Plate A1.2).

During experiments carried out on Merino wethers in Chapter Four, shedding of wool fibres was observed in areas surrounding the sample site as shown in Plate A1.4. The sampling of skin from the cheek site was accompanied by profuse bleeding and removal of the drug by this route should have been enhanced and in this case, an alternative explanation must therefore be

sought. The work of Jefferies (1964), showed that it is possible the cheek skin of the Merino may possess the propensity to exhibit shedding, and this may precipitate the observed response. As shown by the average rate of cell division per follicle, determined in Experiment 4d the rate of wool growth on the cheek is much slower and this may enhance the effectiveness of the drug. For these reasons two successive samples from the cheeks was considered unacceptable, and furthermore, placing sampling sites four centimetres apart proves impossible due to the small area of the cheek.

In conclusion, colchicine was found to be toxic to wool follicle bulb cells and the toxicity at the level of the bulb may relate to the characteristics of the skin. Similarly the effective dose for mitotic arrest is influenced by the skin characteristics and it is therefore imperative to establish dose rates for use in new systems as Tannock (1965) suggested. It is possible that residual levels of the drug are slight after biopsy sampling and that successive samples will be unaffected by this toxicity. The recent work of Hynd and Everett (1990) led to the conclusion that the colchicine technique underestimates the rate of cell division in the follicle bulb. In view of the correlation between cell division rate determined by the colchicine technique and wool growth rate (Schinckel, 1961, 1962; Hynd *et al.*, 1986) it stands to reason that the underestimation of cell division rate is a consistent proportion. Thus, comparison of the number of arrested mitoses in contemporaneous control and treatment biopsies hides the true differences which must of course be greater than those measured.

Modifications to the method such as the change in the vehicle volume have been arrived at by deduction and inference as experience in using the method progressed, and have not yet been rigorously tested. Experimental verification of such minor modifications was viewed as being of little added value to the testing of the major hypotheses of this thesis. The following appendix deals with the statistical procedures which were used in the analysis of results obtained using this method.

Appendix Two.

Statistical Procedures.

A2.1. Introduction.

Previous work involving the intradermal colchicine technique was reported using the median, and non-parametric statistical tests were used because the frequency distributions obtained are usually not normal (Hynd *et al.* , 1986; Scobie and Hynd, 1987). However, there are occasions when a parametric test is more appropriate. For example, when making comparisons between results obtained from a group of biopsies from control animals, with those from a group of treated animals. The reasons for the choice of the different analyses are highlighted below using specific examples. The mean and standard deviation of cell division rate have been used almost exclusively throughout the present thesis, as they were considered to be the most appropriate way of conveying the information, and the basis for this decision is also presented below. The single occasion where medians have been reported was for the purpose of direct comparison with other work, and the word **median** has been highlighted in boldfaced lettering for clarity.

A2.2. Median versus Mean.

The technique of Hynd *et al.* (1986) yields a frequency distribution for the number of cells arrested at metaphase of mitosis in the germinative region of 10 μm sections through the centre of randomly selected follicle bulbs. A typical example of such a random follicle is depicted in Plate 1.1, and below it is the frequency distribution of the population of follicles to which it belongs (see Chapter One). The first thing to notice is that there is a slight bias or skewed nature to the type of data collected in this manner. This is due to the fact that the observations come from a truncated distribution. That is, it is not possible to detect any follicles with a number of mitotic figures less than zero. Indeed, very short periods of arrest are plagued by the nature of such a truncated distribution. Since it is not possible to observe less than zero as a score for cells arrested during division, and there is the chance of observing occasional mitotic figures, by definition the mean will be asymptotic to yet always greater than zero.

For this type of distribution the median may be regarded as a better indicator of the characteristics of the distribution of the number of arrested cells than the arithmetic mean.

However, after compiling many such histograms throughout the course of the experiments it became apparent that the arithmetic mean was a relatively constant amount larger than the median for an arrest period of two hours. This is clearly shown by Figure A2.1, which contains observations from all of the breeds under all of the conditions applied in this thesis, which suggests the median can adequately be described by the function:

$$\text{Mean} = \text{Median} + 0.8$$

In general, over a two hour period of arrest using the optimum dose of colchicine for the breed of animal, this relationship held for populations of follicles from all of the breeds, under all of the treatment regimes studied during the course of the experiments described in thesis. Since it was possible to determine the standard deviation from the data to accompany the mean, the mean was adopted as the standard term in the compilation of tables. The alternative method of expressing the data would have been the use of the median and upper and lower quartiles, in the same manner as the mean and standard deviation.

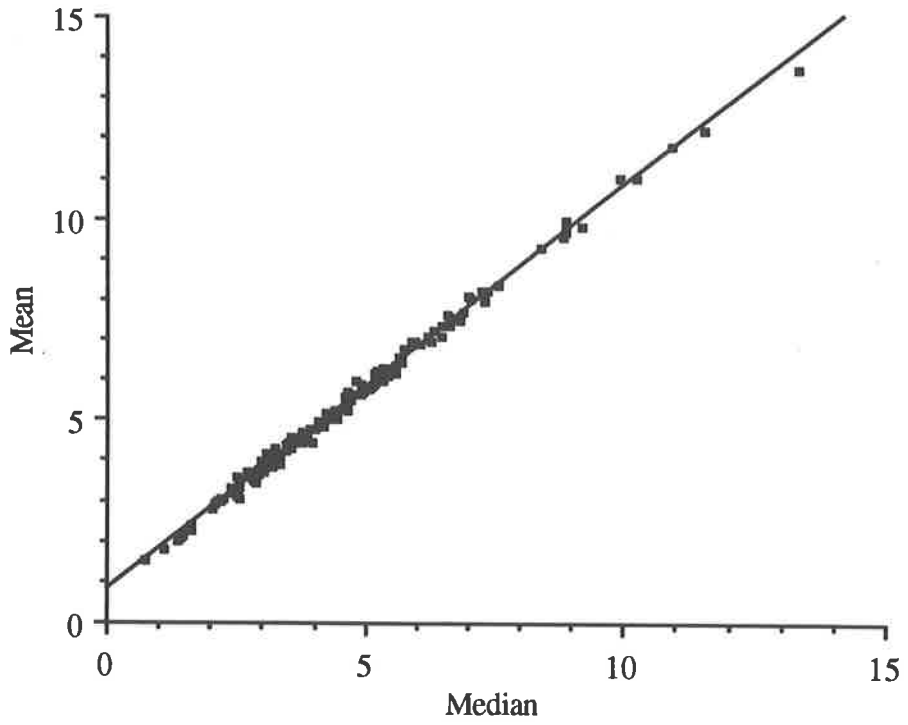


Figure A2.1. The relationship between median and mean for all breeds of animals under all of the conditions imposed during the testing of various hypotheses as outlined in this thesis.

Equation of the line: $y = 0.787 + 1.001x$, $r^2 = 0.996$.

Of course, Figure A2.1 could only be derived after the sequence of events which led to the data assembled in it, and was developed using the observations of the major experiments. It is for this reason that this work has been placed as an aside to the main text, as Appendix One was. This appendix contains a collection of observations on the application and modification of this relatively new technique, as it evolved along with the testing of hypotheses in the main chapters of this thesis. The equation derived from Figure A2.1 provided a rule of thumb by which it was possible to detect samples which may have yielded erroneous data due to an inadequate dose of colchicine, yet were not detected by screening for split mitoses as outlined in Appendix One.

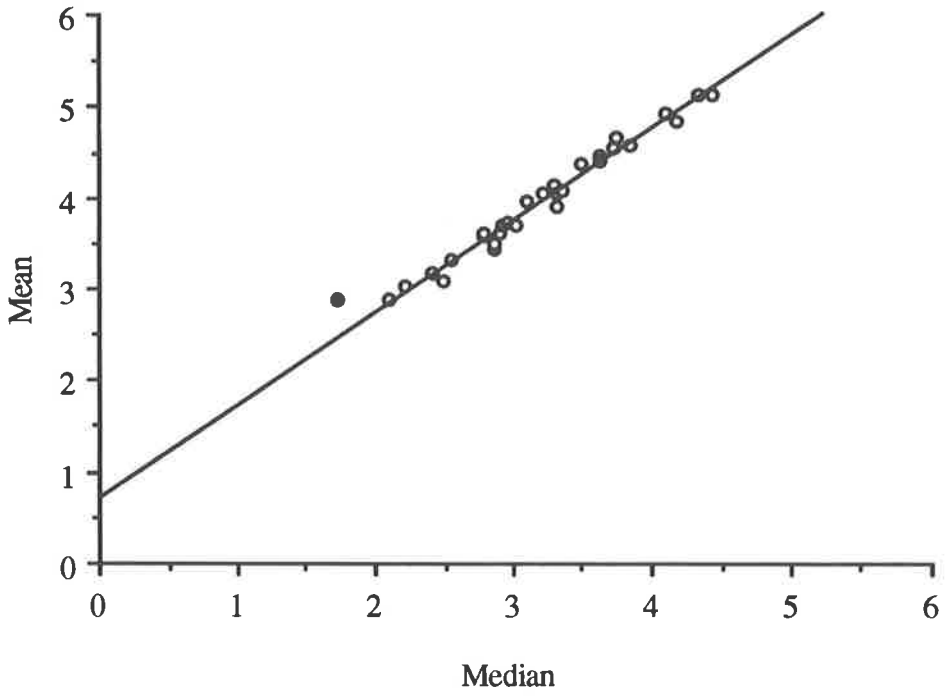


Figure A2.2. The relationship between the median and mean number of cells arrested by colchicine for an adrenalectomised animal (Adx32) prior to adrenalectomy (•), and three sheep post-adrenalectomy (∘) during Experiment 3e.

The simplest way to describe the procedure is by way of an example. The function which describes all the data for samples collected using the standard technique (the method of Hynd *et al.* (1986), 50 µg of colchicine) from Merino wethers during Experiment 3e was: median = mean + 0.71. The data are shown in Figure A2.2, with an additional sample which was collected from animal Adx32 prior to adrenalectomy using the standard technique. Clearly, this

sample does not fit the function described by the other points, the mean was 2.88 yet the median was some 1.16 cells per random bulb section lower. Upon closer examination of an individual frequency histogram obtained from this animal during Experiment 3e (Figure A2.3a) and that of the sample collected from this animal prior to adrenalectomy (Figure A2.3b), we notice that the populations have very differently shaped frequency distributions. The corresponding median and mean (± standard deviation) of each follicle population (n > 200) were; 2.2 and 3.0 (± 1.9) respectively for Figure A2.3a, and 1.7 and 2.9 (± 2.6) respectively for Figure A2.3b. Using the t-test (Sokal and Rohlf, 1981) the populations described by Figures A2.3a and b are not significantly different at the five percent level of significance. In direct contrast to this, using the Kolmogorov-Smirnov test (Sokal and Rohlf, 1981) at the same level leads us to the opposite conclusion. Given our knowledge of the form of the frequency distributions, the non-parametric test is the obvious choice.

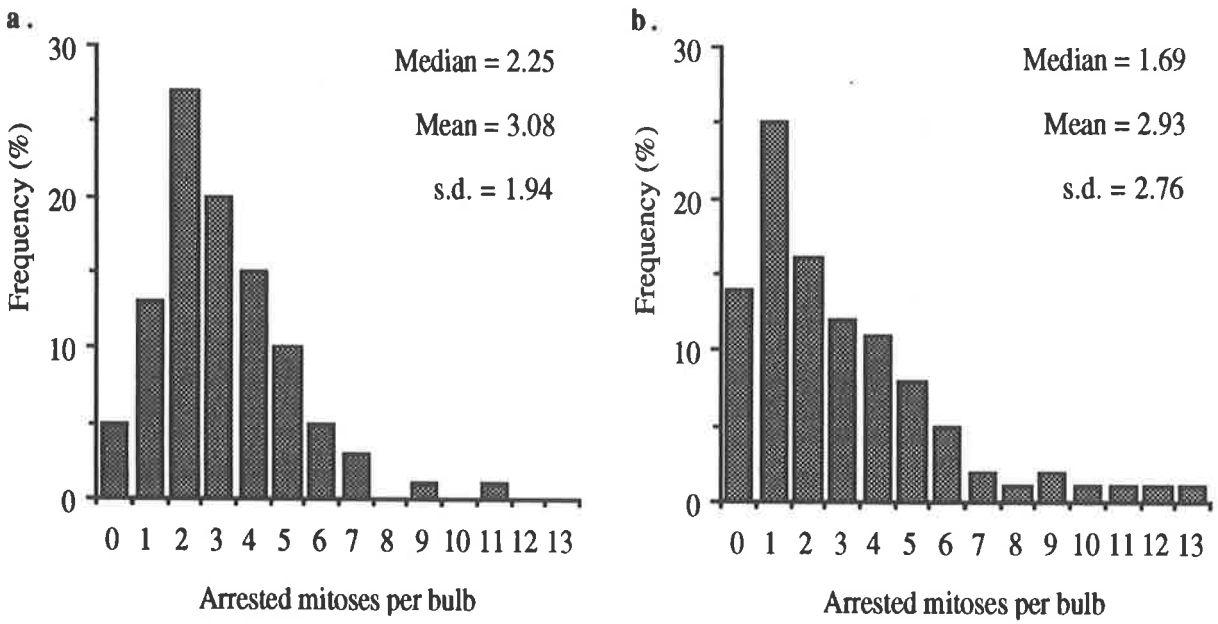


Figure A2.3. Frequency distribution for the number of cells arrested per average random follicle bulb section for animal Adx32; (a) on day 12 of Experiment 3e, and (b) in one sample collected prior to adrenalectomy from this animal.

In biological terms, the statistical problem outlined in the previous paragraph and Figures A2.2, A2.3a and A2.3b equate to the following. In the sample collected from the animal prior to

adrenalectomy there were a large number of follicle bulbs which received an inadequate amount of colchicine to arrest cell division. This was not detected by rapidly screening the samples for split mitotic figures, and further observations suggested that in this case there may have been a poor distribution of the colchicine throughout the skin sample. One half of each histological section exhibited far less arrested mitoses (i.e. a large proportion of 0 and 1 arrested cells per follicle, which was not an effect of poor staining. Such observations were uncommon during all of the experiments of this thesis, however when they were detected the sample was disregarded and treated as a missing value. (It is for this reason that Chapter Three contains no description of methods or results for the samples prior to adrenalectomy and the example has been included here as it clearly shows the different conclusion arrived at using the alternative statistical tests).

Other examples of exceptions to the rule of thumb appear in Appendix One, in the results of experiments used to determine the correct dose rate for a given breed. Cell division rate recorded in samples collected from the Lincoln ewes in Experiment A(ii) were adequately described by the relationship between the median and the mean in the case of the 100 μg dose, but not the 50 μg dose.

Although both parametric (Students t-test) and nonparametric tests (Kolmogorov-Smirnov test) were carried out for all comparisons, the number of follicles counted per treatment was always in excess of one hundred, and there were few occasions where the two tests were discrepant. In the event of a disagreement between the results of the tests, careful consideration was given to the shape of the frequency histogram. Essentially, the Kolmogorov-Smirnov test reveals those histograms which have a different shape, whereas the t-test compares the mean number of arrested mitoses obtained from each histogram.

A2.3. Time Series.

The experiment of Chapter Two was designed to detect whether a circadian rhythm occurs in the rate of cell division in the wool follicle. The choice of design in that experiment can best be summed up by the following quote from McIntosh and McIntosh (1980). "It is very important

for the experimenter to always be aware that internal rhythms of synthesis, sensitivity, clearance, transport rates, concentrations, receptor sensitivity and so on may, and probably do, exist. Measurements on individuals may be essential to detect these rhythms, as data from groups will obscure all but the most striking features." It was for this reason that a small number of animals were sampled frequently in an attempt to emulate the results of Scobie and Hynd (1987). This was also the reason that samples were routinely collected between 1400 and 1800 hours from other animals in all other experiments. Although the results of Chapter Two revealed no distinct circadian rhythm, there was a tendency for cell division to be variable in the morning and more consistent in the afternoon for animals accustomed to being fed in the morning.

The analysis of turning points (Kendall, 1976) used in experiments designed to detect a circadian rhythm was the simplest form of analysis to use in detecting a time series. The non significant result of this test was regarded as sufficient evidence to proceed no further in the analysis of the time series.

A2.4. Analysis of Variance and Covariance.

Although comparisons between samples collected from an individual were limited to nonparametric tests, the means obtained from samples collected from a number of individuals during one experiment were more normally distributed. Thus it was possible to use analysis of variance to determine for example, whether the effect of a given hormone or dose of hormone was significant. Analysis of variance was used in both *in vitro* experiments (Experiments 3b and 4b) and also in analysing fibre length growth in Experiment 5a. In this type of experiment the analysis of variance techniques used were those outlined by (Sokal and Rohlf, 1981), with the calculations being conducted either manually, or using GENSTAT version 5.

The analysis of covariance described in Experiment 3f was conducted because the changes in mitotic rate within an animal were smaller than the differences between animals. Once again, this is best explained using an example. Animal Adx6 and Adx9 were both members of the group infused with the low dose of cortisol, and respectively showed the highest and lowest

rate of cell division throughout the twelve day period. The average number of cells arrested over two hours ranged from 2.94 on day one, down to a low of 2.27 on day seven and returned to 3.25 on day 11 of the infusion in animal Adx6. In animal Adx9 the values were 5.81, 4.23 and 5.97 respectively, almost twice that recorded in animal Adx6. The results could have been adjusted by dividing the daily cell division rate by the average cell division rate of all twelve days. Analysis of covariance is a much more elegant procedure, and Packard and Boardman (1988) recommend that this form of analysis be used in preference to the use of ratios to scale data. These procedures were carried out using GENSTAT version 5, the average cell division rate over the first four days being used as the covariate.

A2.4. Paired t-test.

The results obtained in Experiment 4d (Section 4.5.) were analysed using a paired t-test, since the sympathectomised and unoperated biopsies were treated as matched pairs, and found to be significantly different ($p < 0.026$). A more simple way of analysing the results obtained would be to use probability. A higher rate of cell division in the unoperated ear, was observed in only one animal (SS4) of the nine in the group which exhibited the expected cooling response. Since we expected cell division rate to be lower in the unoperated ear, we can use a one-tailed test. The one-tailed probability of this happening by chance alone is 0.0195, (i.e. significant at the 5% level). In comparison, only four of the nine, or roughly half of the animals in the other group showed a reduced cell division rate in the biopsies taken from the unoperated side, which we would expect by chance. The Kolmogorov-Smirnov test revealed a significant difference in cell division rate in biopsies from unoperated sites of the animals which showed the greatest difference in cell division on one side with respect to the other. Thus in this case, a paired t-test using the means was more sensitive than individual, non-parametric tests.

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