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FACTORS WHICH AFFECT THE FIBRE OUTPUT OF ISOLATED WOOL FOLLICLES MAINTAINED IN VITRO

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

by

Louise Maree Winder

Lincoln University 1995
Factors Which Affect the Fibre Output of Isolated Wool Follicles Maintained in Vitro

by L. M. Winder

A technique for growing individual wool follicles in vitro was developed to test the hypothesis that endocrine growth factors are responsible for the regulation of wool growth. Examination of the fibres produced showed that they were morphologically similar to fibres grown on the sheep, with the rate of wool growth being approximately half of that measured in vivo. This observation was consistent across all sheep breeds examined. Isolated wool follicles continued fibre production for 4.1±0.2 days (mean ± S.E.M.) with a range of zero to twelve days. Follicle culture was therefore regarded as an appropriate in vitro model of in vivo fibre growth.

Follicles grown in culture were used to investigate the mechanisms by which fibre growth is regulated. Wool growth became dissociated from seasonal and nutritional regulation when follicles were isolated from the sheep. Marked seasonal changes (P < 0.001) in in vivo wool growth rates were recorded over a ten month period for English Leicester sheep, but were not observed when follicles from the same sheep were grown in vitro. Similarly, in vivo wool growth responses to increased dietary energy and nitrogen were not carried over into cultured follicles. These results are consistent with the regulation of follicle growth rate being achieved through extrafollicular mechanisms.

The inclusion of adult ovine serum and specific growth factors in the nutrient medium provides further support for the hypothesis that systemic regulation of wool growth occurs. Ovine serum was inhibitory for fibre growth in a season and breed dependent manner. Wool growth inhibition was most apparent in follicles isolated during winter and in follicles supplemented with serum collected during winter. Fibre growth by follicles isolated from an English Leicester sheep was more sensitive to inhibition by ovine serum than growth by follicles from Drysdale sheep. Fractionation of ovine serum enabled preliminary identification of the key inhibitory components as a 55kD protein (possibly albumin), and either a > 350kD protein (possibly α₁-lipoprotein) or an unidentified 130kD protein.

A reduction in the fibre growth rate was also observed following the addition of basic fibroblast growth factor (45% reduction, P=0.003), epidermal growth factor (33%, P=0.05) or transforming growth factor alpha (20%, P=0.16) to the tissue culture medium. At high concentration, minoxidil also inhibited fibre growth (45%, P<0.001). In contrast, insulin,
insulin-like growth factor-1, prolactin, melatonin and aqueous deer antler extract had no effect on the rate of fibre growth.

These results suggest that wool growth regulation by environmental stimuli may be achieved through inhibition of a biochemical pathway. They also indicate that specific growth factors may act on components of this pathway. This study therefore supports the hypothesis that the systemic regulation of wool production occurs through the expression of endocrine growth factors rather than via modification of the wool follicle itself.
TABLE OF CONTENTS

Abstract ii
Table of Contents iv
List of Tables vii
List of Figures x
List of Plates xii
Abbreviations xiv

Chapter 1 Introduction
1.1 General introduction 2
1.2 Nature and scope of this investigation 2

Chapter 2 A Review of the Literature
2.1 Sheep and wool production 7
2.2 Wool fibre and follicle structure 9
2.3 Primary and secondary follicles 15
2.4 Wool follicle initiation 15
2.5 The fibre growth cycle 16
2.6 Wool fibre proteins 17
2.7 Cellular regulation of wool growth and structure 19
2.8 Environmental regulation of wool growth and structure 21
2.9 Growth factor regulation of wool growth and structure 28
2.10 Wool follicle culture 34

Chapter 3 Materials and Methods
3.1 Wool follicle isolation 38
3.2 Wool follicle culture 41
3.3 Measurement of wool fibre growth 43
3.4 Statistical treatment of data 46
Chapter 4  Characterisation of *In Vitro* Fibre Production

4.1 An evaluation of *in vitro* follicle and fibre morphology

4.2 Evaluation of fibre growth in nutrient free medium

4.3 Correlation of *in vivo* wool growth rate with that observed *in vitro*

4.4 Correlation of follicle bulb size with *in vitro* fibre growth rate

4.5 The influence of maintaining wool follicles *in vitro*, on fibre medulla formation

4.6 Discussion and conclusions

Chapter 5  Optimisation of the Culture Conditions for Isolated Wool Follicles

5.1 Introduction

5.2 An evaluation of the culture temperature

5.3 Composition of the basal tissue culture medium

5.4 Supplementation of the basal culture medium

5.5 Supplementation with foetal calf serum

5.6 Discussion and conclusions

Chapter 6  An Evaluation of *In Vitro* Wool Growth for Sheep of Different Genotypes

6.1 Introduction

6.2 Experimental procedure

6.3 Results

6.4 Discussion and conclusions

Chapter 7  An Evaluation of the Effect of Ovine Serum on Wool Growth and Follicle Viability *In Vitro*

7.1 Introduction

7.2 Effects of adult ovine serum on wool growth by follicles maintained *in vitro*

7.3 Fractionation of ovine serum

7.4 Wool follicle culture in the presence of serum fractions

7.5 Discussion and conclusions
Chapter 8  An Evaluation of the Effect of Season and Nutritional Status, Prior to Follicle Isolation, on Wool Production In Vitro 114
8.1 Introduction 115
8.2 Seasonal effect 116
8.3 Nutritional effect 120
8.4 Discussion and conclusions 121

Chapter 9  An Evaluation of the Effect of Melatonin and Prolactin on Wool Production In Vitro 123
9.1 Introduction 124
9.2 Experimental procedure 125
9.3 Results 125
9.4 Discussion and conclusions 127

Chapter 10  An Evaluation of the Effect of Potential Growth Affecting Agents on Wool Production In Vitro 130
10.1 Introduction 132
10.2 Insulin 133
10.3 Insulin-like growth factor 135
10.4 Fibroblast growth factor 137
10.5 Epidermal growth factor 138
10.6 Transforming growth factor alpha 140
10.7 Ethanolamine / phosphoethanolamine 141
10.8 Minoxidil 142
10.9 Antler extract 145
10.10 Discussion and conclusions 147

Chapter 11  General Discussion 150
11.1 Evaluation of the follicle culture technique 151
11.2 Significant outcomes of this study 153
11.2 Potential for future research 158

Acknowledgements 160
Bibliography 162

Appendix A  Statistical analysis of data 189
Appendix B  Polymerase chain reaction 196
Appendix C  Published reports of this study 200
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>Fibre growth and follicle viability following enzyme assisted or manual dissection</td>
<td>41</td>
</tr>
<tr>
<td>3-2</td>
<td>Evaluation of fibre growth and follicle viability when follicles are cut at the sebaceous gland level prior to microdissection</td>
<td>41</td>
</tr>
<tr>
<td>3-3</td>
<td>Details of supplements for which stock solutions were prepared</td>
<td>42</td>
</tr>
<tr>
<td>4-1</td>
<td>Distance between consecutive cuticle ridges on wool fibres from freshly isolated follicles and following maintenance in tissue culture</td>
<td>56</td>
</tr>
<tr>
<td>4-2</td>
<td>Effect of saline or Control Medium A on fibre growth in culture</td>
<td>63</td>
</tr>
<tr>
<td>4-3</td>
<td>The rate of wool production <em>in vivo</em> and <em>in vitro</em> by one English Leicester and one Drysdale sheep</td>
<td>65</td>
</tr>
<tr>
<td>4-4</td>
<td>Details of the fibre diameter and proportion of medulla and pseudo-medulla in fibres grown by follicles isolated from an English Leicester sheep</td>
<td>69</td>
</tr>
<tr>
<td>5-1</td>
<td>Fibre growth by follicles maintained in Control Medium A at 31°C or 37°C</td>
<td>75</td>
</tr>
<tr>
<td>5-2</td>
<td>Wool growth by follicles maintained in different nutrient media</td>
<td>76</td>
</tr>
<tr>
<td>5-3</td>
<td>The vitamin composition of the four nutrient media examined in this study for the <em>in vitro</em> growth of wool fibres</td>
<td>77</td>
</tr>
<tr>
<td>5-4</td>
<td>Fibre growth by cultured wool follicles maintained in William's Medium E with various combinations of supplements</td>
<td>80</td>
</tr>
<tr>
<td>5-5</td>
<td>Effect of microbial contamination of the culture medium, on wool production</td>
<td>82</td>
</tr>
<tr>
<td>5-6</td>
<td>Wool production of follicles maintained in Control Medium A in the presence or absence of foetal calf serum</td>
<td>84</td>
</tr>
<tr>
<td>6-1</td>
<td>Fibre diameter, annual staple length and estimated fibre growth per day for some domestic sheep breeds</td>
<td>87</td>
</tr>
<tr>
<td>6-2</td>
<td>Fibre growth <em>in vitro</em> by follicles isolated from different sheep breeds</td>
<td>88</td>
</tr>
</tbody>
</table>
7-1 Fibre growth rate and follicle viability for follicles isolated from Drysdale or English Leicester sheep and maintained in Control Medium B or serum supplemented medium

7-2 Fibre growth rate a follicle viability of English Leicester follicles isolated at different times of the year and supplemented with 20% frozen English Leicester serum

7-3 Approximate composition of serum fractionation samples following densitometry of the gel shown in Plate 7-1

7-4 Approximate composition of serum fractionation samples following densitometry of the SDS gel shown in Plate 7-2

7-5 Protein concentration of each of the eluant samples

7-6 Summary of the protein composition and purity of the serum fractions produced in this study

7-7 The effect on fibre growth and viability, of maintaining isolated wool follicles in Control Medium B, Control Medium B supplemented with one of the serum fractions defined previously, or physiological saline

8-1 Wool production in vivo and wool growth and follicle viability in vitro, by sheep fed control or energy and protein supplemented diets

9-1 Wool growth by English Leicester follicles in vitro maintained without prolactin or with various prolactin concentrations

9-2 Fibre growth by follicles isolated from New Zealand Wiltshire sheep and maintained in vitro without supplements or with various concentrations of prolactin

9-3 Fibre growth by follicles isolated from Drysdale or English Leicester sheep maintained in vitro without supplements or in medium supplemented with 1.2nM melatonin or 600ng/ml prolactin
10-1 Fibre growth by follicles maintained in insulin free medium or in medium supplemented with various concentrations of insulin
10-2 Fibre growth and follicle longevity for follicles maintained in Control Medium B or in medium supplemented with various concentrations of insulin-like growth factor-I
10-3 Fibre growth by follicles maintained in Control Medium B or in Control Medium B supplemented with various concentrations of basic fibroblast growth factor
10-4 Fibre growth rate of follicles maintained in Control Medium B or in Control Medium B supplemented with various concentrations of epidermal growth factor
10-5 Fibre growth rate by follicles maintained in Control Medium B or in Control Medium B supplemented with various concentrations of transforming growth factor alpha
10-6 Growth of isolated wool follicles maintained in Control Medium B or Control Medium B supplemented with a mixture of ethanolamine and phosphoethanolamine
10-7 Fibre growth rate by follicles maintained in Control Medium B or in Control Medium B supplemented with various concentrations of minoxidil
10-8 Wool growth by isolated follicles maintained in Control Medium B or Control Medium B supplemented with aqueous antler extract
LIST OF FIGURES

2-1 Schematic outline of factors affecting wool production (adapted from White et al. 1979) 7
2-2 Probable relationships of primitive and domestic sheep of the major fleece-type groups showing typical breeds. Adapted from Fraser and Short (1960) 8
2-3 Diagram of sheep skin showing the positioning of fibre follicles (Orwin 1988) 9
2-4 Diagrammatic representation of two wool follicles, sectioned longitudinally showing the different functional zones (Orwin 1988) 10
2-5 Structural representation of a non-medullated and a medullated wool fibre showing the various components of the fibre cortex (Chapman and Ward 1979) 12
2-6 Light microscopic details of a fibre follicle showing the sebaceous gland and follicle bulge (Holbrook and Minami 1991) 17
2-7 The amino acid transulphuration pathway (modified from Reis 1988) 24
2-8 Polyamine transamination (Reis 1988) 25
3-1 Method for determination of follicle longevity in culture 44
3-2 Changes in follicle plus fibre length over time for follicles maintained in vitro 45
3-3 The frequency distribution of the fibre growth rate by follicles isolated from an English Leicester sheep 48
4-1 Relationship between follicle bulb size and fibre growth rate of follicles isolated from one English Leicester sheep and maintained in vitro 66
4-2 Diagrammatic representation of follicle plus fibre following seven days maintenance in tissue culture 68
6-1 Relationship between in vivo and in vitro wool growth by sheep of six different breeds 93
7-1 Major components of blood serum
7-2 Diagrammatic outline of the procedure used to fractionate ovine serum prior to the supplementation of tissue culture media
7-3 Elution profile of large molecular weight serum proteins following DEAE chromatography
7-4 Calculated protein concentrations in each of the eluant samples using biuret ELISA
7-5 Molecular weight standard curve created using the molecular weight markers shown in Plate 7-2
7-6 The hierarchical organisation of pituitary-controlled endocrine growth factors

8-1 Seasonal variation in the rate of wool growth \textit{in vivo} by four English Leicester sheep during the period May 1993 to February 1994
8-2 Growth rate of wool fibres from follicles isolated from four English Leicester ewes and maintained \textit{in vitro}
8-3 Correlation between the rate of \textit{in vitro} and \textit{in vivo} wool growth by wool follicles from four English Leicester sheep
8-4 Fitted and observed relationship for the calculated factor 'Corrected Variation' using a 5\textsuperscript{th} order polynomial

10-1 The rate of wool growth by isolated follicles maintained in Control Medium B or Control Medium B supplemented with different concentrations of antler extract
<table>
<thead>
<tr>
<th>Plate</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>Photograph demonstrating the method for collecting skin strip biopsies using the modified scalpel apparatus</td>
</tr>
<tr>
<td>3-2</td>
<td>The appearance of follicles in skin strips collected with the double-bladed scalpel</td>
</tr>
<tr>
<td>4-1</td>
<td>Photograph of a wool follicle immediately after isolation from the midside of a Romney sheep</td>
</tr>
<tr>
<td>4-2</td>
<td>Photograph of the same follicle as that shown in Plate 4-1, after three days maintenance in Control Medium A</td>
</tr>
<tr>
<td>4-3</td>
<td>A wool follicle which has been maintained in vitro for 48 hours and shows adhesion to the tissue culture plate due to cellular outgrowths from the outer root sheath</td>
</tr>
<tr>
<td>4-4</td>
<td>Electron micrograph of a region of wool fibre produced by an English Leicester sheep prior to isolation of the wool follicle for maintenance in vitro</td>
</tr>
<tr>
<td>4-5</td>
<td>A region of the same wool fibre shown in Plate 4-4 which was synthesised during maintenance of the follicle in vitro</td>
</tr>
<tr>
<td>4-6</td>
<td>BrdU incorporation by cells of the wool follicle bulb following maintenance of the follicle in vitro for three days in Control Medium A</td>
</tr>
<tr>
<td>4-7</td>
<td>Polymerase chain reaction amplification of keratin gene DNA in a longitudinal section of an isolated wool follicle which had been maintained in vitro for 72 hours</td>
</tr>
<tr>
<td>4-8</td>
<td>Transverse section through a wool follicle showing PCR amplification of keratin gene DNA</td>
</tr>
<tr>
<td>4-9</td>
<td>Transverse section through a wool follicle which has been Eosin-y stained only, no gene amplification has been undertaken</td>
</tr>
<tr>
<td>4-10</td>
<td>Transverse section of a wool follicle showing the effect of excluding the enzyme Taq polymerase from the PCR reaction mixture</td>
</tr>
<tr>
<td>4-11</td>
<td>Transverse section of a wool follicle which has not undergone protease digestion prior to keratin gene amplification by PCR</td>
</tr>
</tbody>
</table>
6-1  Histological slide showing follicles in the skin of a Crossbred (finewool) sheep     90
6-2  Histological slide showing follicles in the skin of a Poll Dorset (downwool) sheep     90
6-3  Histological slide showing follicles in the skin of an English Leicester (longwool) sheep     91
6-4  Histological slide showing follicles in the skin of a Drysdale (carpetwool) sheep     91
7-1  Photograph of native gel electrophoresis of protein fractionation samples showing purple bands where protein is present     104
7-2  Photograph of SDS gel following separation of protein fractionation samples showing purple bands where protein is present     105
# Abbreviations Used in This Thesis

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AgResearch</td>
<td>New Zealand Pastoral Agriculture Research Institute Limited</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BrdU</td>
<td>5'-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>ddH_{2}O</td>
<td>distilled, deionised water</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagles medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxyribonucleoside triphosphates</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetate (disodium salt)</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td><em>et al.</em></td>
<td><em>et alii</em>, and others (Latin)</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>H_{2}O_{2}</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>MgCl_{2}</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NZ</td>
<td>New Zealand</td>
</tr>
<tr>
<td>OFDA</td>
<td>optical fibre diameter analyser</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pers.comm.</td>
<td>personal communication</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TGFα</td>
<td>transforming growth factor alpha</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
</tr>
<tr>
<td>WRONZ</td>
<td>Wool Research Organisation of New Zealand (Inc.)</td>
</tr>
</tbody>
</table>
CHAPTER ONE

INTRODUCTION

....the sheep, surpassing however all others if utility be the measure of their value to us, for they are our sovereign protection against the rigours of the cold, generously yielding their soft fleeces for the comfort of our bodies.

L. Jun. Moderatus Columella
First century A.D.
1 Introduction

1.1 General Introduction

1.2 Nature and Scope of this Investigation
INTRODUCTION

1.1 GENERAL INTRODUCTION

Sheep were first introduced into New Zealand by Captain James Cook on his second voyage to the South Pacific in 1773, however the two Merino sheep brought on that occasion failed to survive. Whalers later released small numbers of Merino sheep on several off-shore islands in the New Zealand group and some of these animals have survived as a flock of a primitive Merino strain from Arapawa Island (Simpson, 1990). A small flock of Merino sheep was established on mainland New Zealand by Samuel Marsden in 1814, followed in 1834 by the establishment of a sizeable flock of Merino sheep on Mana Island by James Bell White. By 1880 wool had become the main export commodity of New Zealand.

In 1993, the value of raw wool exports was $NZ 1.3 billion per annum. However competition from synthetic fibres such as nylon, polyester and acrylics has become a challenge to the wool industry due to the flexibility of their processing properties and their relatively low cost of production. This threat to the viability of wool industries has been met by increases in wool production efficiency on farms through improved pastures and pasture management, selection of more productive sheep and improved animal nutrition and health. In addition to improved production efficiency, it is also vital that the wool industry is able to respond to changing market requirements for specific wool characteristics. To achieve this, a clear understanding of the mechanisms that control wool growth and characteristics is needed.

Recent research into the regulation of wool growth has involved nutritional trials with the intradermal and intravenous administration of chemical compounds such as steroid hormones and growth factors. This type of experimentation has frequently succeeded in altering fibre growth, but has rarely resulted in further understanding the factors responsible for such change. Furthermore, such experimentation involves many ethical considerations.

In 1989 researchers at Cambridge, England, developed a technique whereby human hair could be maintained in tissue culture, allowing the mechanisms controlling hair growth and development to be studied (Philpott et al., 1989). The possibility exists that a similar approach could be used to understand the mechanisms controlling wool growth and wool characteristics, if the Cambridge technique could be adapted to culture wool follicles in vitro.

1.2 NATURE AND SCOPE OF THIS INVESTIGATION

The aim of the research described in this thesis was to examine the hypothesis that endocrine growth factors are responsible for the regulation of wool growth. To that end, agents known, or likely, to affect fibre production were identified, and the extent to which these factors act directly on the follicle to influence wool growth and structure, were explored. This was achieved by isolating individual follicles and culturing them in nutrient media in the presence or absence of
the agents of interest. Treatment effects were identified by measuring changes in the rate of fibre growth and follicle viability.

The establishment of a wool follicle culture system and evaluation of wool fibre growth in response to seasonal, nutritional and endocrine challenges are presented in this thesis.
The progress of science is strewn, like an ancient desert trail, with the bleached skeletons of discarded theories which once seemed to possess eternal life.

Arthur Koestler (1905-1983)
British author
2 A Review of the Literature

2.1 Sheep and Wool Production
   2.1.1 The effects of genotype on wool characteristics

2.2 Wool Fibre and Follicle Structure
   2.2.1 Dermal papilla and follicle bulb
   2.2.2 Cortex
   2.2.3 Medulla
   2.2.4 Fibre cuticle
   2.2.5 Inner root sheath
   2.2.6 Outer root sheath
   2.2.7 Connective tissue sheath and glassy membrane

2.3 Primary and Secondary Follicles

2.4 Wool Follicle Initiation

2.5 The Fibre Growth Cycle
   2.5.1 Stem cells

2.6 Wool Fibre Proteins
   2.6.1 Trichohyalin
   2.6.2 Keratin

2.7 Cellular Regulation of Wool Growth and Structure
   2.7.1 Cell to cell interactions
   2.7.2 Trichocyte proliferation and differentiation
   2.7.3 Follicle size and shape
2.8 Environmental Regulation of Wool Growth and Structure  21
  2.8.1 Season  21
  2.8.2 Sheep age and gender  22
  2.8.3 Nutrition  22
    2.8.3.1 Amino acids  23
    2.8.3.2 Vitamins  25
    2.8.3.3 Minerals and trace elements  26
    2.8.3.4 Energy  27

2.9 Growth Factor Regulation of Wool Growth and Structure  28
  2.9.1 Insulin  28
  2.9.2 Insulin-like growth factors  29
  2.9.3 Fibroblast growth factor  30
  2.9.4 Epidermal growth factor  31
  2.9.5 Transforming growth factors  32
  2.9.6 Growth hormone  33
  2.9.7 Glucocorticoids  33
  2.9.8 Ethanolamine and phosphoethanolamine  33

2.10 Wool Follicle Culture  34
  2.10.1 Follicle cell culture  34
  2.10.2 Follicle explant culture  35
  2.10.3 Follicle organ culture  35
A Review of the Literature

The production of a fibre coat distinguishes mammals from other animals and serves a variety of purposes. In addition to affecting appearance, and attraction of the opposite sex, the production of a fibre covering provides insulation and protection against the external environment. The distinction between hair and wool is equivocal, however wool is frequently of a smaller diameter than hair fibres and has a lesser degree of medullation. In this thesis wool is defined as the body coat fibre produced by domestic sheep, and more specifically the predominant fibre type produced by the species *Ovis aries*.

In the years since sheep flocks were established in New Zealand, considerable research has been carried out in an attempt to understand, with a view to controlling, wool growth and wool characteristics. In this chapter, some of this previous work is examined in a review of the literature relevant to the study undertaken for this thesis. Initially, the structure and physiology of follicles and wool fibres is described in detail, while later sections of this chapter present the current state of knowledge regarding the effects of environmental and systemic regulators on wool fibre structure and growth.

The publication of research into the mechanisms of mammalian hair growth began during the 1930's (Barritt *et al.* 1930; Fraser and Roberts 1932). Initially, research concentrated on the effects of administered sulphur amino acids on fibre growth but by the 1940's the work was extended to include the examination of endocrine effects (Whitaker and Baker 1948; Baker and Whitaker 1949). Study of the follicle itself and its relationship to fibre production was initiated in the 1950's by researchers such as Auber (1950), Carter (1955) and Braun-Falco (1958). Tissue culture of skin explants to examine the mechanisms of fibre growth also began at this time. In 1951, Hardy examined follicle development, and later undertook comparisons of the development of mouse fibres in vivo and in vitro (Davidson and Hardy 1952). This research was followed by additional valuable studies into the nature of the follicle (Rogers 1959a,b,c; Rogers 1964; Rogers and Clarke 1965).

Enormous volumes of literature have since been produced on fibre production and structure, however until recently, there remained a dearth of information on the mechanisms by which changes in fibre growth may be effected. This has partly been due to the limited availability of experimental techniques to study the activity of the fibre follicle (Frater and Hewish 1981; Messenger 1985; Philpott *et al.* 1991). Recent advances in follicle cell and keratinocyte culture bode well for future progress (Philpott *et al.* 1989; Philpott *et al.* 1990; Holbrook and Minami 1991; Chuong *et al.* 1991). In vitro systems allow for the rapid, inexpensive study of a large variety of substances, as well as replacing animal testing (Lingna *et al.* 1991). Furthermore, follicle cell culture techniques provide a tool for identifying the processes controlling the fibre growth cycle and keratin synthesis (Frater and Hewish 1981). Identification of growth factor and drug actions at the cellular level will be facilitated by the development of these new techniques (Messenger 1985).
2.1 **Sheep and Wool Production**

Sheep are warm blooded ruminants, efficient at the conversion of pasture into wool which is essential for the maintenance of body warmth. Over the centuries, sheep have been bred for increased wool production and improvement in the characteristics that influence its use by man for insulation and decoration. These characteristics are dependent on the dimensions and composition of individual wool fibres and the manner in which fibres interact in the fleece and during manufacturing into woollen goods.

Wool is formed from keratin proteins and the type of keratin synthesised is believed to be determined genetically, as fibre protein composition varies between different breeds of sheep (Ebling 1976; Allden 1979; Marshall and Gillespie 1989). Non-genetic factors also play a role however, as keratin synthesis varies between individual sheep within one breed, between fibres at different locations on the one animal, and in regions of the same fibre produced at different times of the year. It appears that the animal's genetic potential for wool production is seldom realised due to the interactions of nutrition, hormonal status, climate and disease which limit wool productivity (Robards 1979). These factors affect the animal's ability to synthesise many body proteins efficiently and result in changes in wool fibre characteristics (Kempton 1979). Wool growth can also be altered as a result of cellular malfunctions in the wool follicle due to ageing, genetic change, dietary manipulation or the administration of chemicals (Chapman 1988).

A simple outline of factors which interact and affect wool production is presented in Figure 2-1 (adapted from White et al. 1979). The quality and composition of pasture available to each animal regulates the supply of amino acids and other nutrients to the wool follicle. Anti-nutritive substances and toxins in the pasture, for example endophytic fungal alkaloids, can affect the health of the animal and so reduce wool protein synthesis (Thompson and Stuedemann 1993). The nutritional requirements of an animal for maintenance, pregnancy, lactation and growth, affect nutrient partitioning and result in changes in wool production and concomitant wool characteristics (White et al. 1979). Factors which affect the viability of the wool follicle itself, for example blood flow, temperature and physical damage, also have the potential to regulate wool production (Marshall and Gillespie 1989; Thompson and Hynd 1994).

![Figure 2-1 Schematic outline of factors affecting wool production (adapted from White et al. 1979).](image-url)
While effects on wool growth of many of the factors described in Figure 2-1 have been demonstrated, a full appreciation of the controls of wool growth is likely to occur only by understanding the activity and regulation of the wool follicle itself (Chapman and Ward 1979; Oliver and Jahoda 1988). It therefore seems likely that the development of new concepts with the potential for improving and/or controlling wool production, with a view to regulating fleece characteristics, will be enhanced by a basic knowledge of the factors which affect wool protein synthesis (Booth 1988). This is also likely to require an understanding of gene activity within the wool follicle bulb, the site of cell proliferation and differentiation (Bawden et al. 1987). The growth of wool follicles in tissue culture, in isolation from environmental influences, has the potential to provide answers to some of these basic wool growth questions. This observation has led to the current study.

2.1.1 THE EFFECTS OF GENOTYPE ON WOOL CHARACTERISTICS

In the previous section it was noted that sheep genotype has a considerable influence on wool fibre characteristics and the rate of wool production. This is reviewed extensively by Allden (1979). Wool produced by domestic sheep breeds is classified into four main groups of phenotypically isolated fibre types; carpetwool, longwool, downwool and finewool. Figure 2-2 is an adaptation of that presented by Fraser and Short (1960) and outlines possible evolutionary pathways of primitive and domestic sheep breeds.

![Diagram of probable relationships of primitive and domestic sheep of the major fleece-type groups, showing typical breeds, adapted from Fraser and Short (1960).]

The capacity of a sheep to grow wool is set by its genotype (Nagorcka 1979; Panaretto 1979; Reis 1988) as this largely determines the number and characteristics of the wool follicle such as density, size and curvature (Allden 1979; Black and Reis 1979; Black 1987). Sheep genotype also influences the efficiency of nutrient partitioning between the skin and other tissues (Allden 1979; Black and Reis 1979; Black 1987), a mechanism correlated with wool growth differences (Daly and Carter 1955; Allden 1979). An examination of the influence of sheep genotype on wool follicles is presented in Chapter Six of this study. Follicles were isolated from animals of
seven sheep breeds and maintained in tissue culture to identify whether genetic differences in fibre regulation occur at the wool follicle or are due to systemic factors.

2.2 WOOL FIBRE AND FOLLICLE STRUCTURE

The development of a research programme to identify factors involved in the regulation of wool production necessitated a review of the current state of knowledge with regard to wool follicle and fibre structure. This review of key findings is presented here and provides a background for the structural examination of wool fibres grown in vitro presented in Chapter Four.

The wool follicle is a highly specialised, complex organ located within the dermal tissue of the sheep, as shown in Figure 2-3. Individual wool follicles are surrounded by connective tissue with the follicle bulb in close proximity to the adipose layer.

Figure 2-3 Diagram of sheep skin showing the positioning of fibre follicles (Orwin 1988).

The structure of all hair and wool follicles is similar, and has been extensively reviewed (for example Orwin 1979a; Chapman and Ward 1979; Orwin 1988). From a functional viewpoint the follicle may be considered to consist of a number of longitudinally and radially differentiated cell types. Figure 2-4 was produced by Orwin (1988) and shows the specific zones of activity along the length of follicles containing fibres with and without a medulla. Details of the cellular activity occurring within these zones is also presented below.
Zone A The mitotic zone is also variously termed the germinative region of the bulb and the proliferative zone. It consists of the dermal papilla and the base of the follicle bulb which consists of rapidly dividing and differentiating cells.

Zone B Cellular elongation occurs in this zone, following differentiation. Zones A and B constitute the follicle bulb.

Zone C In this region wool protein synthesis occurs in differentiated cells.
Zone D  Keratinization (the process of disulphide bond formation and crosslinking of keratin proteins) occurs in this region.

Zone E  In this region the final hardening of the fibre occurs and degradation of follicle cell layers immediately adjacent to the fibre begins.

Zone F  This zone occurs just below the level of the sebaceous gland duct where there is considerable breakdown of the inner root sheath cell layer.

Zone G  The sebaceous gland opens into the pilary canal surrounding the formed fibre in this region which continues up to the skin surface.

The different cell types within the wool follicle form structures with specific, defined functions in wool growth. These follicle structures are shown in Figure 2-4 and are further described below.

2.2.1  DERMAL PAPILLA AND FOLLICLE BULB

The follicle bulb is located at the base of the follicle (Zones A and B, Figure 2-4) and is the site of the mitotically active cells involved in wool production (Chapman and Ward 1979). As shown in Figure 2-4, the dermal papilla is invaginated into the follicle bulb (Jahoda et al. 1984). The dermal papilla is frequently pear-shaped, however a number of variations in shape have been noted, and are thought to be modulated by changes in nutrition or season (Auber 1950) although possible mechanisms have yet to be elucidated. Strong correlations between dermal papilla and bulb size occur (Straile 1965; Henderson 1965; Cohen 1965; Ibrahim and Wright 1982; Orwin 1988; Kelly et al. 1993) and this is discussed in further detail in Section 2.7.3 of this chapter. Fine-woollen breeds tend to have follicles of shorter length and narrower diameters than coarse-woollen breeds.

The dermal papilla is the mesenchymal component of the follicle bulb with the remainder being formed from epithelium. The dermal papilla is primarily composed of specialised fibroblasts with associated collagen. Blood vessels are also typically present in the dermal papilla and enable the distribution of nutrients and regulatory factors within the follicle (Orwin and Woods 1982). The presence of blood vessels are restricted to the papilla so alternate mechanisms for nutrient dispersal within the follicle bulb must exist. Follicle cell membranes are replete with gap junctions, pores in the membranes through which low molecular weight molecules can pass directly (Orwin and Thomson 1972; Manchester 1976; Marshall et al. 1991). These are important for the dispersion of nutrients, and between breed variations in the distribution of these junctions may affect wool characteristics and growth (Orwin 1980).

Dermal papilla cells normally show very low proliferative activity and are considered to be predominantly mitotically inactive (Orwin 1979b; Messenger 1984). The dermal papilla is separated from the highly mitotically active bulb cells by a basement membrane which is continuous with the connective tissue sheath (Orwin and Woods 1982). Wool growth results from the intense proliferative activity of matrix cells within the follicle bulb (Kiesewetter and Schell 1994). The dermal papilla is thought to regulate cell division and differentiation within the bulb by the production of intercellular signals in response to external stimuli (Messenger et al.
1991). These signals are believed to pass through the basement membrane and ultimately control mitosis in the follicle bulb (Messenger et al. 1991).

The dermal papilla is essential for normal fibre growth (Cohen 1965; Orwin 1988; Arase et al. 1994). Fibre growth does not occur in the absence of the dermal papilla (Oliver and Jahoda 1988), and transplanted papillae can induce hair follicle formation (Orwin 1979b; Young 1980; Reynolds and Jahoda 1991). The dermal papilla therefore has the potential to regulate fibre formation although evidence of regulation through altered papilla activity, intercellular signals or morphology have yet to be documented.

### 2.2.2 Cortex

The major product of most wool follicles is the fibre cortex (MacKinnon et al. 1990) which consists of cells filled with keratin protein. As described in Figure 2-5, the cortex is formed from elongated cells filled with protein bundles called macrofibrils. These are the main structural components of the cortex, although cortical cells also contain degraded nuclei and organelle remnants (Rogers 1959b; Chapman and Ward 1979). Macrofibrils are formed from fibrous protein structures which were historically termed microfibrils, but are presently referred to as intermediate filaments (Gillespie and Marshall 1988; Marshall et al. 1991). The macrofibrils consist of intermediate filaments occurring as a coiled-coil helical structure packed together either as a two-strand or three-strand rope, and a non-fibrous proteinaceous matrix (intermediate filament associated protein) which acts as a cementing material (Rogers 1959b,c). The intermediate filaments and their associated proteins interact by disulphide bonds and hydrophobic linkages (Rogers 1991).

![Diagram of fibre cortex](image)

**Figure 2-5** Structural representation of (a) a non-medullated and (b) a medullated wool fibre showing the various components of the fibre cortex (Chapman and Ward 1979).
Two principal types of cortical cells have been described; orthocortical and paracortical cells, however cells of intermediate morphology (meso- and metacortical cells) have also been reported (Powell et al. 1988; Orwin 1988; Orwin and Bailey 1988; Powell et al. 1991). The different cell types vary in keratin protein composition and in the packing arrangement of the intermediate filaments (Orwin and Bailey 1988; Marshall et al. 1991). In orthocortical cells the filaments are in a whorl-like pattern, as if they are coiled around each other, while in paracortical cells they tend to be in hexagonal quasicrystalline arrays, as if they are straight and lying parallel to each other (Rogers 1959c; Bawden et al. 1987; Rogers 1991).

The factors affecting the production of different cortical cell types and the controlling mechanisms have yet to be determined although it is thought that the distribution of cortical cell types must originate during cell differentiation in the wool follicle bulb (Bawden et al. 1987). In addition, the importance of the cortical composition of wool with regard to its performance during processing and in end products, has yet to be fully defined however bilateral segmentation of ortho- and paracortical cells has been linked to the phenomenon of crimp which is associated with wool bulk (Powell et al. 1988). There is also some evidence that cell type proportions may influence tensile properties such as strength (Orwin et al. 1980; Orwin and Bailey 1988). Effects of genotype, season and nutrition on cuticle cell type is currently undefined.

2.2.3 Medulla

Many wool fibres do not contain a medulla, however when one does occur it is typically found in the coarser fibres of the fleece (Orwin 1988). A medulla occurs in the central core of the fibre cortex, as can be seen in Figure 2-5. It is composed of cortical cells which do not produce keratin proteins but rather synthesise trichohyalin protein granules which, during fibre development, form a hardened, amorphous structure (Rogers 1959a; Orwin 1979a; Rothnagel and Rogers 1986).

A number of medulla types have been described, from continuous through to interrupted. There appears to be a correlation between dermal papilla:bulb volume ratio, and medulla formation, however the controlling factors regulating formation of a medulla have yet to be identified (Auber 1950). The medulla is an important structural component of wool fibres as the presence of a medulla significantly affects fibre characteristics and processing properties (Orwin 1979b). Appreciable differences in the degree of medullation occur between breeds of sheep and genetic, nutritional, seasonal and disease factors have each been implicated in affecting the degree of medullation (Orwin 1979b).

2.2.4 Fibre cuticle

The fibre cuticle consists of cells composed of amorphous protein and is typically only a few cells thick (Rogers 1959a). The differentiation pathways of cuticle cell formation have recently been detailed (Powell et al. 1991), although control mechanisms have yet to be identified. The external scale pattern of the fibre cuticle differs along the length of fibres (with season and nutrition) and between breeds (with genotype), however changes in cuticle cell composition in
concert with this variation have yet to be examined. The importance of the cuticle scale pattern is demonstrated during industrial processing of the fibre. Variations in the fibre scale pattern such as scale size and ridge height are associated with propensity to felting during processing and the lustre of the resultant textile product (Woods and Orwin 1982; Orwin and Woods 1983). In addition, although the fibre cuticle is highly impermeable and appears to protect the cortex of the fibre (MacKinnon et al. 1990), its composition and integrity determines the ability of the fibre to absorb water and dyes during yarn manufacture.

2.2.5 Inner root sheath

Immediately surrounding the wool fibre are three layers of cells which comprise the inner root sheath. These cell layers are termed the inner root sheath cuticle, Huxley's layer and Henle's layer (Chapman and Ward 1979) and are composed of microfibrillar protein (Rogers 1959a). All three cell types are degraded and sloughed into the pilary canal in the upper follicle (Orwin and Woods 1985). The roles of Henle's and Huxley's layers in fibre formation are not well defined (Orwin 1988). Henle's cells comprise the outermost layer of the inner root sheath and form a hardened tubular structure which may provide support for the development of other cell lines (Straile 1965). Cells in the Huxley's layer usually differentiate and harden later than Henle's cells (Auber 1950). They frequently form in a single layer around the inner root sheath cuticle, however they may become multilayered, resulting in significant variations in inner root sheath thickness, in response to seasonal and other unidentified factors (Priestley and Rudall 1965; Woods and Orwin 1982).

The inner root sheath cuticle consists of cells which form scales that point downward from the skin surface and these interlock with similar but opposing scales which form the surface of the fibre. This close-fitting interaction suggests that the fibre and inner root sheath move together during fibre growth (Straile 1965). The inner root sheath cuticle is thought to dictate the fibre cuticle scale pattern as ridges in the fibre cuticle occur at the edges of inner root sheath cuticle cells, rather than at those of the fibre cuticle (Woods and Orwin 1982; WRONZ 1982). Stabilisation of the fibre cuticle pattern occurs due to covalent cross-linking of the cuticle proteins (Woods and Orwin 1980) however details of the cellular mechanisms giving rise to variations in the cuticle scale pattern have yet to be elucidated.

2.2.6 Outer root sheath

Like all cellular components of the wool follicle, the outer root sheath cells arise from mitosis of cells within the bulb and are the only follicle cell line continuous with the epidermis (Auber 1950; Orwin 1971). In wool production terms, little is known about the role of the outer root sheath in fibre growth, although the migration of sheath cells towards the skin surface is thought to assist movement of the wool fibre (Straile 1965). In addition, a characteristic of many cells in this layer is their high content of glycogen, which suggests an energy storage role for the outer root sheath (Braun-Falco 1958).
2.2.7 **Connective Tissue Sheath and Glassy Membrane**

Surrounding the outer root sheath are a glassy membrane, also called the hyaline or vitreous membrane, and a connective tissue sheath (Montagna and van Scott 1958), all of dermal origin (Chapman and Ward 1979). The glassy membrane consists of a basement membrane, adjacent to the outer root sheath and two thin layers of collagen fibres, the inner being oriented along the follicle and the outer encircling the follicle. As noted previously, a continuation of the basement membrane encloses the dermal papilla within the bulb. The connective tissue sheath consists of a further two, thicker layers of collagen fibres and fibroblasts (Orwin 1988). These layers are also arranged at right angles to each other, but in the reverse order to those in the glassy membrane. These follicle components are likely to provide structural support and integrity to individual wool follicles.

2.3 **Primary and Secondary Follicles**

Two types of wool follicles have been defined. These are termed primary and secondary follicles depending on their time of initiation in foetal skin. Primary follicles are formed in the foetus at approximately 60 days post-conception, with secondary follicles being initiated approximately 14-20 days later (Hardy 1951; Carter and Clarke 1957a,b; Orwin 1988). Primary follicles are typically larger and produce fibres of a greater diameter than secondary follicles (Orwin 1988; Hardy 1992), although relative size differences between primary and secondary follicles are breed-related. In Chapter Six of this study, isolation of wool follicles from sheep of different genotypes has shown the importance of primary and secondary follicle size in relation to the ease of follicle isolation from the skin and their successful maintenance in vitro.

Wool follicles are arranged in a regular pattern in the skin, usually in groups, with one large primary follicle flanked by smaller secondaries (Hardy 1992). There is a large genetic component in the proportion of secondary:primary follicles (S:P ratio). Finewool sheep breeds such as the Merino have a high proportion of secondary follicles and therefore a much higher S:P ratio (~20) than that of longwool or carpetwool breeds (~5) (Orwin 1988).

2.4 **Wool Follicle Initiation**

In the early sheep embryo, wool follicles are derived from the ectoderm, an epithelial layer that will give rise to the epidermis, and the underlying mesoderm, a mesenchymal layer that will form the dermis (Hardy 1992; Panaretto 1993). Reorganisation of the basement membrane which separates the ectoderm and mesoderm always precedes follicle initiation with epithelial cells adjacent to the basement membrane becoming oriented with their long axes perpendicular to the membrane and cells destined to become the outer root sheath having their long axes parallel to the basement membrane (Hardy 1951). The fibre follicle begins as a down growth of a small group of basal epithelial cells into the mesenchyme. As the epithelial follicle bud elongates, its base surrounds the mesenchymal cluster which will form the dermal papilla (Frater and Hewish 1980; Rogers et al. 1987). Development of the follicle proceeds with the differentiation of the epithelial cells into multiple layers (Sutton et al. 1995). A wool fibre then begins to grow from
the epithelial cells next to the dermal papilla, the bulb matrix cells, by rapid cell division, elongation and synthesis of keratin proteins (Panaretto 1993).

2.5 THE FIBRE GROWTH CYCLE

The growth pattern of wool is typically cyclic, with follicles proceeding from an active phase, through a regression and shortening phase, to a resting phase. In wild animals there are frequently differences between the cycles of primary and secondary follicles, which implies local control of individual follicles (Hardy 1992).

In domesticated sheep breeds, most follicles are in the anagen (active) phase of growth for much of the year (Orwin 1988; Powell et al. 1991), however hair follicles located on the extremities of the sheep frequently undertake regular cyclical activity and shedding (Hutchinson 1965). In these follicles, and in sheep breeds which do produce cyclical fibre growth and fleece shedding (eg New Zealand Wiltshire), anagen is followed by a transitional phase (catagen). In this state a club-like structure, sometimes referred to as a 'brush-end' forms at the base of the developing fibre, and the follicle reduces in length (Panaretto 1979). Catagen is followed by a dormant stage (telogen), during which development of a new fibre is initiated following replication and differentiation of follicle stem cells (discussed in 2.5.1) (Philpott et al. 1990). As the hair follicle cycles throughout the lifetime of the animal, a process similar to embryonic follicle initiation occurs each time the telogen hair follicle is induced to re-enter anagen. As it grows, the anagen hair follicle migrates through the dermis and it has been proposed that collagenases and other proteases may be important in follicle invasion in the deeper dermis, as well as in the remodelling observed during involution of the hair bulb in catagen (Weinberg et al. 1990). The potential for growth factor involvement in this process is examined in Chapter Ten of this thesis.

The initiation and maintenance of fibre cycles is likely to involve the dermal papilla as it remains intact during catagen and re-initiation of the fibre. In addition, the proportion and composition of the extracellular matrix within the dermal papilla varies throughout the fibre growth cycle (Couchman et al. 1991; Ashcroft et al. 1994). During anagen, the dermal papilla contains a high proportion of extracellular matrix which is rich in the basement membrane proteins fibronectin and proteoglycan (glycosaminoglycan covalently bound to a core protein) (Moretti et al. 1967; Butler 1975; Maekewa 1979; Frater and Hewish 1981; Jahoda et al. 1987). The proportion of these matrix proteins is reduced in catagen and almost non-existent during telogen when the papilla appears as a tightly packed ball of cells (Messenger et al. 1991). The significance of cyclic changes of glycosaminoglycans within the wool follicle is currently unknown, however they have effects in a number of biological systems and it has been suggested that they may provide some type of immunological protection for follicle growth (Messenger et al. 1991). Further identification of the regulation of papilla morphology will result in a better understanding of the factors affecting wool growth and fibre cycling. Steroid hormones have been implicated as possible control agents, however this has been based on studies using non-physiological doses of hormones, and these have yet to be conclusively linked with hair growth cycles (Mohn 1958; Panaretto 1979; Philpott et al. 1990).
2.5.1 **Stem Cells**

Stem cells exist in all self-renewing tissues. They are a long-lived cell populations exhibiting a great potential for cell division and are intimately linked with the processes of tissue renewal, wound healing, aging and carcinogenesis (Lavker et al. 1991; Reynolds and Jahoda 1991). In order to better understand the factors controlling wool follicle viability, it is important to identify the stem cell populations because of the controlling effect these may have on follicle activity.

Stem cells are relatively undifferentiated and give rise to rapidly proliferating, transiently amplifying cells following growth stimuli or wounding. In most tissues, stem cells are found in well protected, highly vascularised and innervated locations (Lane et al. 1991). It has been suggested that in the wool follicle, the bulge or Wulst region (Figure 2-6) of the outer root sheath would fulfil these criteria (Holbrook and Minami 1991). In support of this hypothesis, mitosis was detected in cells of the bulge region only during early anagen, confirming that they proliferate transiently, solely at the onset of anagen, and strongly supports the suggestion that bulge cells are the origin of the whole lower follicle in anagen (Wilson et al. 1994).

![Figure 2-6](image)

**Figure 2-6** Light microscopic details of a fibre follicle showing the sebaceous gland (SG) and follicle bulge (B) (Holbrook and Minami 1991).

2.6 **Wool Fibre Proteins**

The cortical cells of mature wool fibres are filled with tightly packed keratin and trichohyalin proteins (Bawden et al. 1987; Marshall et al. 1991) which are produced following the expression of genes for these proteins in the elongation and keratinization zones (zones B, C and D in Figure 2-4). Keratin gene expression is initiated in cuticle and cortical cells, whereas trichohyalin gene activation occurs in medulla and inner root sheath cells (O'Guin et al. 1989;
McGuire et al. 1980). All wool proteins are characteristically high in sulphur amino acids. The amino acid cystine is the predominant form of sulphur in wool, although cysteine and methionine are also present. Wool is also rich in glutamic acid, serine and glycine (Reis 1979).

The sulphur content of wool varies considerably, depending on the type of keratin protein produced. As discussed in Section 2.1 of this chapter, genetic and nutritional factors, in addition to the physiological state of the animal, affect wool protein synthesis and therefore the characteristics of the wool fibre and its processing properties (Reis 1979; Woods and Orwin 1987; Marshall et al. 1991). The protein composition between, and along the length of individual wool fibres can be highly variable (Frenkel et al. 1974; Marshall and Gillespie 1989; MacKinnon et al. 1990). The development of a technique for growing wool follicles in tissue culture may enhance our ability to identify the specific mechanisms which regulate keratin and trichohyalin protein synthesis, and therefore the characteristics of wool important for industrial processing. This is one of the aims for the further development of the research presented in this thesis.

2.6.1 Trichohyalin

Inner root sheath and medulla cells both synthesise trichohyalin (Auber 1950; Woods and Orwin 1982), a protein unique to these cell types (O'Guin and Sun 1987). Trichohyalin is distinguished from other wool proteins by the presence of the non-dietary amino acid citrulline which is derived by a post-translational modification of arginine residues (Rogers 1959a; Rogers 1963; Rogers and Clarke 1965; Rothnagel and Rogers 1986). In the inner root sheath, trichohyalin is an intermediate filament associated protein that promotes the alignment and aggregation of intermediate filaments (O'Guin and Manabe 1991). It may also have a role in the separation of the inner root sheath from the emerging wool fibre at the sebaceous gland level as trichohyalin is highly sensitive to protease activity (O'Guin and Manabe 1991). In the medulla, trichohyalin granules enlarge and coalesce during fibre maturation, however contrasting reports have been presented regarding the final structure of the medulla. Rogers et al. (1991) report that trichohyalin granules grow and eventually fill medullary cells with amorphous protein, whereas in their review, Marshall et al. (1991) suggest the granules form a hardened mass around vacuolar spaces within the cells of the medulla.

2.6.2 Keratin

In the sheep over 100 distinct proteins are synthesised by the hair cortex and cuticle cells to produce the keratin structure of wool fibres (Hardy 1992). The keratins can be divided into two major groups, the intermediate filament proteins and the intermediate filament associated or matrix proteins (Woods and Orwin 1987; Marshall et al. 1991). These groups may be further subdivided, on the basis of their most abundant amino acids, into the low sulphur $\alpha$-helical keratins, which form the intermediate filaments, and the high sulphur, ultra-high sulphur and high glycine-tyrosine keratins, of which the filament associated proteins are formed (Rogers et al. 1989; Powell et al. 1991).
Keratin gene transcription is rapidly activated following cell differentiation in the follicle bulb (Bawden et al. 1987; O'Guin et al. 1989; MacKinnon et al. 1990; Powell et al. 1991). Activation of all keratin genes does not occur in unison, there is a sequential and spatial expression pattern for each of the gene families within the follicle and the developing wool fibre (Powell et al. 1991). Synthesis and deposition of intermediate filaments appears to occur prior to the generation of the filament associated proteins (Woods and Orwin 1987). Disulphide cross-linking, or keratinization, both within and between protein chains then converts the protein structure into a stable, insoluble form (Orwin 1976a). At the same time, catabolic changes result in the lysis of cell organelles, their associated protein and nucleic acids (Gillespie and Marshall 1988).

2.7 Cellular Regulation of Wool Growth and Structure

It has been discussed earlier, in Section 2.1.1, that follicle size varies according to sheep breed, and that this affects the size of the wool fibre produced. In addition to follicle size, there are a number of other structural components of the wool follicle that have been correlated with changes in fibre production, and specific details on the regulation of fibre production by these factors are discussed here. The rate of proliferation and differentiation of wool forming cells (trichocytes), interactions between cells, and the ultimate size and composition of these cells, determines the morphology of the wool fibre.

2.7.1 Cell to Cell Interactions

In wool follicles the epidermal germinative matrix cells are separated from the dermal papilla by the basement membrane which supports a number of gap junctions (Hardy 1992). Direct contact between the two cell types has been observed through these gaps which suggests signal transmission between the dermal papilla and hair matrix cells may occur by this mechanism (Hardy 1992; Buffey et al. 1993). Receptor-mediated signalling is a further likely mechanism for the interaction of these two cell types during wool fibre formation.

Regulation of fibre synthesis via interaction with cells of the dermal papilla is likely to involve extracellular matrix molecules which are synthesised in the dermal papilla and include heparin sulphate proteoglycans (Young 1980; Fritae et al. 1985; Hardy 1992), fibronectin (Norman et al. 1989), laminin (Norman et al. 1989; Panaretto 1993) and epimorphin (Panaretto 1993). Matrix molecules bind to specific cell surface receptors and to each other with adhesion being dependent on cell type (Norman et al. 1989). Involvement of these extracellular matrix components in the provision of information and instructions for cells, dictating their attachment, migration, proliferation and differentiation has been documented (Hardy 1992). Fibre regulation may be achieved by altering the patterns of gene expression and cellular function of cells in the matrix of the wool follicle bulb (Norman et al. 1989). Cell to cell interactions are therefore critical to the maintenance of viable wool follicles, and dermal papilla - matrix interactions are likely to be one mechanism by which fibre morphology is regulated.
2.7.2 **TRICHOCYTE PROLIFERATION AND DIFFERENTIATION**

In this thesis, changes in fibre length will be taken as the measure of fibre growth, and continued fibre elongation will be the measure of follicle survival. These simplistic measures belie the fact that numerous cellular processes underlie these measurable components.

Cell proliferation occurs when there is sufficient active DNA synthesis, and cell division, to increase the number of cells. An increase in cell size occurs as part of the cell cycle prior to DNA synthesis, but in some cell types, cellular hypertrophy, defined as an increase in the protein content of a cell without DNA synthesis, may be the terminal response to a particular stimulus (Norman *et al.* 1989). This phenomenon is typical of cells involved in the production of a wool fibre. Mitosis occurs within the follicle bulb with cellular elongation and hypertrophy occurring once the cell has moved from the mitotic zone. No further mitotic activity is undertaken by these trichocytes (Auber 1950).

Wool fibre growth and structure vary with dermal papilla size (Hynd 1994), the number and size of cells in the proliferative zone of the follicle bulb (Williams and Winston 1987; Kelly *et al.* 1993; Hynd 1994), the rate of division of bulb cells (Downes *et al.* 1966; Chapman and Ward 1979; Franke and Held 1988) and the proportion of newly formed cells that migrate from the proliferative zone and enter the fibre (Fraser 1965; Wilson and Short 1979; Hynd 1989a,b). The size of cells forming the fibre (Chapman and Ward 1979; Hogan *et al.* 1979; Orwin 1980; Orwin and Woods 1983) and the rate of cell death also influence the characteristics of the resultant fibre (Orwin 1976b). These factors are determined genetically but are believed to be affected by nutrition and other environmental factors, however more research is required to understand how they interact to vary follicle morphology in relation to fibre production and properties (Orwin 1988).

Some interesting work has been done regarding the fate of trichocytes produced in the follicle bulb. A few studies have suggested that any one mitotic cell may have the capacity to differentiate into any of the cell types present in the bulb, that is they may be pluripotential. However it is more likely that trichocyte differentiation is predetermined, or restricted according to its position in the bulb (Auber 1950; Orwin 1971; Woods and Orwin 1982; Hynd 1989b). This suggests that changes in factors which alter trichocyte proliferation, such as nutrition, do not alter cell commitment or cell type once division has concluded.

2.7.3 **FOLLICLE SIZE AND SHAPE**

A correlation has been described between fibre growth rate and follicle length, and fibre volume with follicle volume. Small follicles produce less wool due to a reduction in the volume of mitotic cells, rather than any effect of less efficient cell kinetics (Williams and Winston 1987; Hynd 1994). Bulb mitotic activity and volume have been shown to vary with seasonal and nutritional changes (Wilson and Short 1979; Hynd *et al.* 1986; Orwin 1988), however this does not always occur (Williams and Winston 1987) and details of the mechanisms regulating fibre production through altered follicle size have yet to be determined.
In addition to follicle length, differences in follicle curvature and the angle the follicle lies in the skin significantly affect follicle depth and fibre shape. Genetic influences are primarily involved, although in some instances follicle depth varies with nutrition and season (Orwin 1988). It appears that the shape of the dermal papilla confers much of the shape of the resultant fibre due to its control of follicle bulb size and shape (Auber 1950; Wilson and Short 1979).

2.8 ENVIRONMENTAL REGULATION OF WOOL GROWTH AND STRUCTURE

The environment in which an animal is maintained affects wool production. This observation was presented briefly in Section 2.1 of this chapter and some aspects are discussed in detail here.

2.8.1 SEASON

Most domesticated sheep breeds show little evidence of fibre shedding, however a correlation between wool growth rate and fibre diameter, with season, have been widely reported (Coop 1953; Bennett et al. 1962). The influence of season on fibre morphology is currently receiving considerable attention, although a causal relationship has not been established and breed-specific differences occur. Changes in season precipitate changes in a number of factors which may influence wool production, namely temperature, photoperiod, pasture quality and animal physiology (for example pregnancy and lactation).

Seasonal changes in photoperiod provide a likely candidate for the regulation of fibre growth as in most sheep breeds, a reduction in fibre diameter is correlated with reduced photoperiod (Nagorcka 1979). Little is currently understood about the mechanisms of this light induced effect, however the endocrine hormones melatonin and prolactin have been implicated (Panaretto 1979; Morgan 1990) although they have also been refuted (McCloghry et al. 1992b; Curlewis 1992). Effects of these factors on the follicle remain to be conclusively determined and are examined experimentally, using isolated wool follicles, in Chapter 9 of this study. The regulation of follicle viability and fibre growth, by seasonal influences, are also examined in Chapter 8.

Seasonal changes in temperature appear less likely to be responsible for cyclic regulation of wool growth as exposure of a well-fleeced sheep to cold temperatures does not result in changes in wool production (Bottomley 1979). Nonetheless, correlations of temperature changes with altered wool production have been documented. Shearing, and consequential exposure of the sheep to a reduction in temperature, results in an increase in wool growth rate (Hopkins and Richards 1979) and fibre diameter (Bottomley 1979). This may be due to increased nutrient availability to the wool follicles, as an increase in feed intake following shearing has been reported (Bottomley 1979).

Increased feed consumption may result from changes in the activity of the hypothalamus, as temperature-sensing and feeding loci are believed to be in close proximity. Changes in hypothalamic expression may result in altered synthesis of thyroid hormone; changes in which have been demonstrated to confer feed intake and wool growth modifications during exposure to cold (Hopkins and Richards 1979). Exposure of sheep to a prolonged heat treatment results in a
decreased fibre diameter, most likely through suppression of feed intake (Bottomley 1979). Exposure of a small area of skin to cold reduces fibre growth rates in the exposed area due to a reduction in length but no change in diameter (Coop 1953; Bennett et al. 1962; Wallace 1979; Hopkins and Richards 1979). This may occur due to a reduction in skin blood flow (Setchell and Waites 1965). Induction of glucocorticoids due to stress may also be involved, as a decrease in wool growth has been demonstrated in response to increased glucocorticoids (Wallace 1979).

These findings suggest that wool production may be affected by changes in temperature, however whether this effect is regulated at the wool follicle, or is mediated by nutritional changes with consequential alteration in endocrine hormone status, has yet to be determined. In this study, effects of altered follicle temperature, on wool growth in vitro, are examined in Chapter 5.

2.8.2 Sheep age and gender

Wool growth rate and fibre characteristics such as diameter, colour and crimp vary with sheep age (Chapman and Jackson 1972; Hogan et al. 1979). Maximum fleece weights are typically recorded at three to four years of age followed by a decline due to a reduction in follicle viability and volume (Corbett 1979).

Rams produce more wool than ewes of a similar age, however rams typically have a greater bodyweight and therefore a greater skin surface area for wool growth; the fibre output from each wool follicle is likely to be similar (Corbett 1979). Testosterone, an androgen produced by the ram testes, has been shown to stimulate body growth with consequent increases in wool growth. However it is unlikely that testosterone increases wool production independently of effects on feed intake and body growth (Ferguson et al. 1965; Manchester 1976).

A number of studies have demonstrated that wool growth is decreased by pregnancy and lactation (Ferguson et al. 1965; Hogan et al. 1979; Corbett 1979). While feed intake of the ewe typically increases during pregnancy, partitioning of the available nutrients into wool is also likely to be compromised due to the demands of the developing foetal and mammary tissues (Corbett 1979; Black and Reis 1979). Wool growth during pregnancy and lactation may also vary due to changing hormonal status, however no correlation exists between fibre growth and changes in adrenocortical activity or plasma cortisol levels during this period (Ferguson et al. 1965).

2.8.3 Nutrition

A positive relationship between wool growth and feed intake was first demonstrated by Weber, (1931) and has been noted by many others since (Allden 1979; Faichney and Black 1979; Wallace 1979; Aziz et al. 1991). Changes in nutrient availability appear to directly affect mitosis within wool follicle bulb cells (Wilson and Short 1979; Hynd 1989b; Hynd 1994) and the dimensions of the follicle bulb and dermal papilla (Hynd 1994). Cell proliferation, differentiation, commitment and size each appear to be affected, however the correlation of these with fibre morphology is poorly defined (Reis and Downes 1971; Wilson and Short 1979; Allden 1979).
Sheep are ruminants and therefore the rumen microbial population affects the conversion of feed into nutrients (Weston 1979; Falchney and Black 1979; Higgins et al. 1988). In the rumen, carbohydrates are converted to volatile fatty acids which are the main energy supply for sheep. Proteins are hydrolysed and constituent amino acids degraded to volatile fatty acids and ammonia (Falchney and Black 1979). Digestion of any remaining herbage fragments and rumen microbes is undertaken in the small intestine which provides amino acids, carbohydrates and long chain fatty acids (Kempton 1979; Leng 1988). The availability of amino acids for the synthesis of wool keratin proteins appears to be the major limiting factor for wool production (Kempton 1979), however adequate vitamins and trace elements are also essential, as is a sufficient supply of energy to the follicle. Differences in the rate of wool growth between high and low wool producing animals may be due to a greater efficiency of amino acid metabolism by high wool producers (Clark et al. 1989) or to a greater efficiency of conversion of food to wool (Cronje and Smuts 1994).

2.8.3.1 AMINO ACIDS

Amino acid availability, with respect to both type and quantity, is the primary nutritional factor affecting wool growth and fibre characteristics (Reis and Colebrook 1972; Kempton 1979). In particular the sulphur amino acids, methionine and cyst(e)ine, have a vital role in the regulation of wool growth (Reis and Schinckel 1963; Reis 1965; Reis 1979; Pisulewski and Buttery 1985; Higgins et al. 1988; Marshall and Gillespie 1989). Maintenance of wool follicles in vitro, in the absence of individual amino acids, has demonstrated that lysine, methionine, cyst(e)ine, leucine and glutamine are essential amino acids for continued fibre production (Nancarrow and Hynd 1994). As these were the only amino acids evaluated, others may also be essential.

Cysteine has a direct inductive effect on gene expression and increases the rate of transcription of specific ultra-high sulphur genes (Rogers et al. 1991). Cysteine is also required for the synthesis of coenzyme A and may therefore influence the mitotic activity of follicle bulb cells (Reis 1988). As noted in section 2.6, wool keratins are defined as sulphur amino acid containing proteins, therefore sulphur amino acids are essential for keratin synthesis (Downes 1961). An increase in the availability of sulphur amino acids to the wool follicle results in an increased sulphur content in wool fibres due to synthesis of the high and ultra-high sulphur keratin proteins (Gillespie and Reis 1966; Marshall and Gillespie 1989; Harris et al. 1994). However dietary supplementation of sulphur amino acids is ineffective at stimulating wool production due to rumen degradation (Reis 1979; Baldwin et al. 1991), although some techniques whereby amino acids are protected from degradation have been described (Reis and Schinckel 1963). Cystine is synthesised in the skin by the transulphuration of methionine and serine, a pathway detailed in Figure 2-7 (Downes et al. 1964; Hoey et al. 1984; Egan et al. 1984; Pisulewski and Buttery 1985).
Figure 2-7  The amino acid transulphuration pathway (modified from Reis 1988).

Approximately 75% of available methionine is thought to be converted to cysteine (Reis 1988). Omission of methionine from a balanced diet results in decreased fibre growth (Reis and Tunks 1978), diameter (Reis and Downes 1971) and strength (Reis 1988). In addition to effects on the transulphuration pathway, protein synthesis in the inner root sheath may also be affected as the methionine content of this root sheath is substantially higher than in the wool fibre itself (Rogers 1964; Reis 1988). Methionine is also thought to have additional specific effects on wool growth through the functions of S-adenosyl-methionine. S-adenosyl-methionine acts as a methyl donor in the transamination pathway (Figure 2-8), which results in the synthesis of the polyamines spermine, spermidine and putrescine (Rogers 1959a; Reis 1988; Benevenga et al. 1983; Jarvis et al. 1990).
The polyamines are involved in membrane stabilisation, regulation of nucleic acid and protein synthesis, post-synthetic protein modification and are especially important in actively dividing cell systems such as the wool follicle bulb (Williams-Ashman and Canellakis 1979). Polyamine oxidase, an enzyme circulating in ruminant plasma, catalyses the oxidative degradation of spermine and spermidine. The activity of this enzyme, when serum is used to supplement tissue culture studies, may cause polyamine oxidation and lead to the production of hydrogen peroxide (Williams-Ashman and Canellakis 1979). Effects on the rate of fibre growth by isolated wool follicles, of bovine and ovine serum are examined in Chapters 5 and 7 of this study respectively.

In addition to the sulphur amino acids, there are large amounts of glutamic acid, glycine, proline, serine and tyrosine in wool proteins and a number of these amino acids affect wool formation (Hynd 1989b). Glutamic acid, glycine and cysteine are the constituent amino acids of glutathione. The latter is believed to have an important role in cell division (Reis 1988). Diets lacking in lysine reduce trichocyte proliferation and fibre diameter, although length growth rate is increased (Reis and Tunks 1978; Allden 1979). This may arise from a change in commitment of trichocytes and hence an increase in the proportion of migrating cells forming a fibre.

The observed effects of various amino acids on wool growth may be mediated by the synthesis of protein growth factor components of the endocrine system. Some amino acids, for example arginine, have been shown to stimulate the release of growth factors, such as growth hormone and insulin in sheep (Reis 1979). Growth hormone and the thyroid hormone thyroxine have been shown to influence wool growth (Ferguson et al. 1965; Clark et al. 1989). In order to clarify the effects of nutrition on wool growth, it is important to define which endocrine factors affect wool production, and to identify their mode of action in the cells of the follicle bulb.

2.8.3.2 Vitamins

Deficiencies of a number of vitamins have been reported to impair wool growth (Orwin 1980; Chapman and Black 1981; Chytil 1983; Reis 1988). Vitamin A (retinoic acid) is a fat soluble
A deficiency of any of the water soluble B vitamin group appears to affect the distribution of the different cortical cell types described in Section 2.2.2. This results in a marked change in the amount of fibre crimp (Orwin 1980). A reduction in fibre growth rate has also been noted with deficiencies of some vitamins in this group (Chapman and Black 1981). Folic acid, an additional water soluble vitamin, may also be important for wool production due to its role in the remethylation of homocysteine to methionine, as noted in Figure 2-7 (Reis 1988). No evidence has yet been provided for a direct role of folic acid in the regulation of wool growth.

Vitamin D (1,25-dihydroxyvitamin D₃), a fat soluble vitamin, is probably a paracrine hormone rather than a vitamin as it is synthesised by the epidermis which also appears to be its site of action (Epstein 1983). Vitamin D exerts its cellular effects via binding to a high-affinity receptor. Expression of the vitamin D receptor in human hair follicles has been identified in the nuclei of the follicle bulge, the putative site of follicle stem cells (Reichrath et al. 1993). Intense labelling of the vitamin D receptor in the dermal papilla was also observed (Reichrath et al. 1993). Vitamin D has also been localised in the outer root sheath of human hair follicles (Epstein 1983). Harmon and Nevins (1993) found using a follicle culture system, that physiological levels of vitamin D are required for normal hair function.

In Chapter 5 of this study, isolated wool follicles were maintained in one of four different tissue culture media. These media varied in their vitamin composition, therefore effects of some vitamin deficiencies on wool growth in vitro were able to be examined.

### 2.8.3.3 Minerals and Trace Elements

A number of trace elements have been reported to affect wool growth (Purser 1979), however only copper and zinc appear to directly modify fibre formation, whereas other minerals influence feed intake and rumen microflora (Reis 1988).

Copper deficiency leads to reduced wool growth with a reduction in crimp and increased lustre. This is due to a reduction in sulphur incorporation (Reis 1965) and faulty keratinization, as copper catalyses disulphide bond formation and therefore keratin protein crosslinking (Purser 1979). The availability of molybdenum and sulphur regulates the effectiveness of copper (Paynter 1984).
Zinc deficiency results in a reduction in wool growth. The mechanism appears to involve impaired keratinization of the wool fibre rather than a reduced rate of mitosis in bulb cells (White et al. 1994). The resultant fibre has a reduced diameter and is brittle with no crimp (Purser 1979). Zinc may be necessary for the formation of wool keratin or may be a component of an enzyme for wool protein synthesis. It is a co-factor for DNA polymerase and is required for DNA synthesis and is therefore essential for cell replication and wool growth (Reis 1988). Zinc may also be required for the metabolism of cystine and affect wool growth via this pathway (Hsu 1976).

Sodium, as sodium chloride, also affects wool production. This was shown to be due to a decrease in rumen microbial protein degradation, resulting from an increased water intake leading to rapid movement of feed through the rumen (Purser 1979). No direct effects of sodium on wool growth have been demonstrated.

Selenium is essential for the proliferation of a number of epithelial cell types, and sodium selenite is frequently incorporated in cell culture systems. Selenium is a co-factor for glutathione peroxidase which protects cells from peroxide damage (Norman et al. 1989). Selenium also appears to be important for the activation of glutathione reductase which is essential for oxygen metabolism (Maurer 1986). A reduction in wool productivity has been associated with selenium deficient sheep. Selenium supplementation of deficient animals can increase both greasy and clean fleece weights, through increases in fibre diameter rather than yield (Donald et al. 1994). This effect may, however be due to differences in the rumen metabolism of fatty acids or amino acids, or to increased feed intake (Donald et al. 1994). Selenium deficiency usually coexists with iodine deficiency and may further impair thyroid hormone synthesis. Indeed, one of the major effects of selenium deficiency is on peripheral thyroid hormone metabolism by directly affecting the thyroid gland (Zhu and Boyages 1993).

Calcium is also required for normal cell growth. In epidermal cells, keratin cross-linking via epidermal transglutaminase is inhibited when calcium is deficient, as is the activity of calmodulin, which modulates the activation of several enzymes (Thompson et al. 1985).

In this study the effects on wool growth, of supplementing isolated wool follicles with various minerals and trace elements, are examined in Chapter 5.

2.8.3.4 Energy

The wool follicle consists of rapidly proliferating cell populations undertaking extensive protein synthesis and consequently having a high energy requirement.

The major fuel used by isolated human hair follicles is glucose although glutamine is also important (Kealey et al. 1994). A high concentration of glycogen, the storage form of glucose, has been identified in fibre follicles (Black and Reis 1979; Reis 1988). The mechanism by which glucose influences the supply of energy to follicles has yet to be elucidated however the glucose transporters, GLUT1 and GLUT4, are likely to be involved in the process. The GLUT4 glucose transporter isoform is insulin dependent and has a major regulatory role in many tissues, whilst
GLUT1 is an insulin independent transporter (Smith *et al.* 1992; James 1993). Both transporters have been found in wool follicles (Dijkstra *pers.comm.*). As noted in 2.8.3.2, retinoic acid, a vitamin which affects fibre growth, up-regulates gene transcription of GLUT4 (Sleeman *et al.* 1993). It is possible that this mechanism is important in wool production.

It seems likely that the volatile fatty acids which are produced during rumen fermentation are a major energy source (Chapman and Ward 1979), however no glucose-fatty acid cycle operates in the hair follicle (Kealey *et al.* 1994) and lipid fuels such as palmitate or β-hydroxybutyrate have been shown to yield little energy for fibre growth.

### 2.9 Growth Factor Regulation of Wool Growth and Structure

In this study, the term growth factor refers to chemicals which demonstrate a regulatory role on cell proliferation and include steroid hormones, polypeptide hormones, and polypeptide growth factors.

Growth factors may achieve this physiological regulation through one or a combination of autocrine, paracrine or endocrine mechanisms. Autocrine control implies the simultaneous production of and response to a factor by the same cell, paracrine growth factors have effects in tissues surrounding the site of synthesis, and endocrine factors are synthesised at a site spatially separate from the location at which its effects are generated (Norman *et al.* 1989). Growth factor regulation of cell proliferation, growth and differentiation is complex. A balance is achieved within the system between positive and negative regulation; under different circumstances the same factor may affect different cell functions in different ways. Autocrine, paracrine, and endocrine feedback loops, both positive and negative, also operate in the regulation of cell growth (Norman *et al.* 1989).

Growth factor effects on the cell are mediated by binding of the mitogen to an appropriate receptor (Wahl and Carpenter 1987). Receptor molecules may either be bound to the plasma membrane or located intracellularly. Peptide growth factors bind to specific membrane receptors, and the interaction triggers a cascade of intracellular molecular events culminating in cell growth. In contrast, steroid hormones diffuse across the cell membrane and bind to intracellular receptors. The hormone-receptor complexes accumulate in the nucleus and bind directly to DNA to regulate transcription of specific genes (Norman *et al.* 1989). A growth factor's activity may be changed by altering the rate of synthesis of the mitogen itself or changes in the sensitivity, synthesis or availability of it's specific receptor.

The effects of a number of growth factors on wool growth have been reported in the literature and are further evaluated in Chapter 10 of this study.

#### 2.9.1 Insulin

Insulin is an endocrine polypeptide growth factor which is synthesised and released from the pancreas in response to a high blood glucose concentration (Sainz *et al.*, 1990). The binding of circulating insulin to its specific cell membrane receptor on muscle and fat cells initiates a cascade
of cellular events which stimulates the transport of glucose across the plasma membrane. Receptor activation due to insulin binding also results in the activation of glycogen synthetase, inhibition of lipolysis and promotion of protein synthesis from amino acids. These pathways result in an enhanced conversion of blood glucose to glycogen and lipids and increased protein synthesis (Lehninger 1975).

These cellular effects of insulin implicate this growth factor in the regulation of wool production. A high concentration of glycogen, a storage form of energy, has been identified in wool follicles (Braun-Falco 1958; Black and Reis 1979; Reis 1988). This suggests that factors with the potential to affect follicular uptake of glucose and glycogen storage may have a regulatory role in wool follicle activity. Effects of insulin on the stimulation of protein synthesis (Lehninger 1975) also suggest a regulatory role for insulin on the synthesis of wool proteins.

A role for insulin in the regulation of wool production is not however supported by in vivo studies. Wynn et al. (1988) showed that following growth hormone administration, the plasma concentration of insulin was increased and that this was correlated with a reduction in the diameter of wool fibres produced. However, following cessation of growth hormone treatment, the plasma insulin concentration returned to a basal level whereas significant increases in wool fibre length and diameter were observed at this time. In contrast to these observations, Johnsson et al. (1985) found that growth hormone administration to lambs increased fleece weight in concert with a raised plasma concentration of insulin. Bray et al. (1990) showed that wool growth responses to changes in nutrition can be independent of insulin responses. These results provide additional evidence that insulin does not have a major role in the regulation of wool production.

Insulin shares a high degree of structural and functional homology with the insulin-like growth factors. Insulin is believed to act via the insulin-like growth factor-I receptor when present at high concentrations (Philpott et al. 1994; Saintz et al., 1990).

### 2.9.2 Insulin-like Growth Factors

The insulin-like growth factors (IGFs), also termed somatomedins, are small peptides which circulate in the blood and promote diverse biological activities including insulin-like metabolic effects, cell proliferation and cell differentiation (Gilmour et al. 1988; Dickson et al. 1991). Two forms of IGF have been identified, IGF-I and IGF-II, along with two IGF receptors. Type one IGF receptors preferentially bind IGF-I but also bind IGF-II and to a lesser extent insulin. The type-II IGF receptors respond preferentially with IGF-II, bind IGF-I with reduced affinity, and do not bind insulin (Gilmour et al. 1988; Norman et al. 1989).

IGF-I is synthesised and secreted primarily by the liver and circulates in plasma bound to specific carrier proteins, interacting with target tissues via its specific receptors. It has been proposed that IGF-I synthesis is under growth hormone control (Salmon and Daughaday 1957; Dickson et al. 1991). IGF-I mRNA transcripts have been localized in liver tissue and up to tenfold increases in liver transcripts have been documented following growth hormone treatment of animals.
(Saunders et al. 1991). Nonetheless IGF-I mRNA has also been shown to be synthesised in a growth hormone independent manner (Stevenson et al. 1992; Perks et al. 1992) with the nutritional and hormonal status of the animal modulating IGF-I gene expression (Gilmour et al. 1988; Stevenson et al. 1992). In addition, sites of IGF-I synthesis other than the liver have been documented (Stevenson et al. 1992; Perks et al. 1992; Little et al. 1994; Sutton et al. 1995).

A number of studies have shown that IGF-I mediates many of the effects of growth hormone via the endocrine system (Salmon and Daughaday 1957; Dickson et al. 1991; Stevenson et al. 1992; Perks et al. 1992). Through feed-back mechanisms, IGF-I also inhibits growth hormone secretion from the pituitary, and directly inhibits growth hormone gene transcription (Lara et al. 1994).

There is limited information on the role of the IGFs in skin. The major cell type in the epidermis of the skin is the keratinocyte and this cell type has been shown to possess IGF-I receptors and respond to IGF-I (Murashita et al. 1993). These cells do not synthesise IGF-I but appear to respond to growth factors originating from dermal fibroblasts (Sutton et al. 1995) although recent work has identified IGF-I gene transcripts in anagen rat hair follicles (Little et al. 1994). The role of IGFs in the hair follicle is not known (Philpott et al. 1994). IGF-I receptors have been identified in the wool follicles of New Zealand Wiltshire sheep, with greater IGF-I binding observed in germinal matrix than in dermal papilla cells (Nixon et al. 1994a), binding of IGF-II was high in both germinal matrix and dermal papilla (Nixon et al. 1995a). In wool follicles, upregulation of IGF receptor numbers occurred following a hormonal stimulus, prior to observed growth responses (Nixon et al. 1995a). Some observations suggest a regulatory relationship exists between prolactin and IGF in wool follicles. Increased plasma prolactin concentrations upregulate IGF receptor abundance (Nixon et al. 1995a) and may have a role in regulating the activity of the follicle by controlling the transition from anagen to catagen and telogen.

Treatment of pituitary cells with IGF-I resulted in increased prolactin secretion but not in prolactin gene transcription (Lara et al. 1994). IGF-I infusion in sheep increased skin blood flow, amino acid uptake (Harris et al. 1993) glucose clearance, protein synthesis and reduced protein catabolism (Breier et al. 1993). No direct effects of IGF-I have been found on replicating cell numbers in the bulbs of wool follicles (Harris et al. 1993).

2.9.3 Fibroblast Growth Factor

The fibroblast growth factor (FGF) family is composed of at least seven heparin-binding proteins which exist in a wide variety of tissues (Obana and Ito 1993) and are characterised by their potent mitogenic effects on cells and their ability to promote migration and differentiation in various cell types (du Cros et al. 1993). Acidic (aFGF) and basic (bFGF) forms of this growth factor have been documented. Fibroblast growth factors act primarily on cells of mesodermal origin and bind to specific membrane receptors (Norman et al. 1989; du Cros et al. 1993). Seven forms of the FGF receptor have currently been cloned (Panaretto 1993). These receptors
contain a conserved amino acid sequence common to the cell-adhesion molecules, cadherins, and may form part of the cell-adhesion recognition system in concert with their role as growth factor receptors (Panaretto 1993).

Basic FGF, also known as heparin binding growth factor-2, is mitogenic for keratinocytes in vitro (Norman et al. 1989) although in sheep, aFGF was found to be more mitogenic than bFGF for skin keratinocytes, and both forms of FGF elicit proliferative responses in dermal fibroblasts (du Cros et al. 1993). Fibroblast growth factors are expressed in the skin of sheep throughout development of this tissue (Sutton et al. 1995) with keratinocyte synthesis of this growth factor having been documented (Buffey et al. 1993). Fibroblast growth factor receptors have been identified in the developing dermal papilla and hair bulb of mice (Panaretto 1993). Some studies suggest that binding to heparin-like molecules is a prerequisite for the interaction of bFGF with its high-affinity receptor (du Cros et al. 1993) and that this growth factor is synthesised locally within the skin and deposited extracellularly in the basement membrane. The presence of bFGF in the outer root sheath of the fibre follicle and in the basement membrane adjacent to the proliferative zone of the follicle bulb, may indicate specific growth-promoting functions for this growth factor. Bound to the basement membrane, bFGF could provide a continuous proliferative stimulus to the matrix cells and thus directly influence the rate of fibre growth (du Cros et al. 1993). Basic FGF may mediate dermal papilla-matrix cell interactions through the activation of collagenases, as it restored activation of procollagenase in co-cultures of dermal papillae with hair follicles lacking a dermal papillae (Scandurro et al. 1993). Both FGFs stimulate cell proliferation and differentiation in cultured follicle cells (Obana and Ito 1993) although a lack of effect of bFGF on DNA synthesis in follicle bulb cells has been reported (Imai et al. 1992).

2.9.4 **Epidermal growth factor**

Epidermal growth factor (EGF) is a proteoglycan with potent mitogenic effects on numerous cell types (Norman et al. 1989). These effects are mediated via binding of the growth factor to a transmembrane glycoprotein receptor (O'Keefe et al. 1982; Hardy 1992), and transduction of the EGF signal to the nucleus appears to occur via accumulation of the receptor-growth factor complexes in the nucleus, raising the possibility of a direct interaction between this complex and DNA (Norman et al. 1989). Receptors for EGF have been detected in numerous tissues including keratinocytes (O'Keefe et al. 1982) and the epithelial components of hair and wool follicles (du Cros et al. 1992).

*In vitro*, EGF is secreted by cultured dermal fibroblasts and may be synthesised in skin tissues. EGF has been detected in the dermal papilla (Panaretto 1993), sebaceous glands and outer root sheath of fibre follicles, primarily in the zone of keratinization (du Cros et al. 1992). Although the outer root sheath does not keratinise, it may act as a source of EGF to promote keratinization and hardening of the inner root sheath and fibre (du Cros et al. 1992). Both sites of EGF localization in sheep skin, the outer root sheath and sebaceous glands, are regions rich in glycogen. A function for EGF in glycogen synthesis is indicated, although this role is not one of
proliferation, as follicle cells that are mitotically active lack both glycogen and EGF (du Cros et al. 1992).

In vivo, wool production appears to be inhibited by EGF. This is dependent on the concentration, mode of administration and the period of delivery of the growth factor (Hynd 1989b; Moore 1982). Following inhibition of wool growth due to EGF, changes were observed in the keratin composition of early regrowth fibres. The same keratin proteins were present but in different proportions. There was an increased content of high sulphur proteins, particularly the ultra high sulphur proteins, and almost complete suppression of high tyrosine proteins (Frenkel et al. 1975; Gillespie et al. 1982). Administration of exogenous EGF to sheep also resulted in a reduction in the depilation force and strength of wool fibre bundles (Behrendt et al. 1993a,b) and frequently in the rapid shedding of the entire fleece (Gillespie et al. 1982; Moore 1982).

Epidermal growth factor has also been found to have slight mitogenic effects on follicles maintained in culture (Rogers et al. 1987; Weinberg et al. 1990), although Philpott et al. (1991) reported that EGF had no significant effect on hair follicle length after five days in culture. Imai et al. (1992) reported that EGF slightly inhibited the DNA synthesis of hair bulb cells. The most striking influence of EGF, was to enhance collagenase activity, which may be involved in depilation through disruption of the basement membrane and collagen sheath (Rogers et al. 1987). Interestingly, the dermal papilla lacks the type I collagen that predominates in mature dermis (Hardy 1992).

A specific mitogen such as EGF, which could stimulate cell proliferation and enhance proteolytic enzyme activities, would be a powerful physiological regulator for hair growth and activity.

2.9.5 Transforming growth factors

The transforming growth factors (TGFs) are members of the epidermal growth factor (EGF) family of polypeptide growth factors. Two distinct classes of TGFs have been identified, the alpha and beta forms. TGFα binds to the cell membrane EGF receptor triggering the EGF pathway of receptor activity, stimulating mitogenesis in epithelial cells such as keratinocytes (Panaretto 1993; Wynn et al. 1989). Both forms of TGF are multifunctional and may stimulate or inhibit growth depending on the cell type and the presence and activity of other growth factors (Norman et al. 1989).

Transforming growth factor-α is synthesised and secreted by normal adult keratinocytes and the addition of this growth factor, or EGF, to keratinocytes in vitro induces TGFα gene expression suggesting autocrine regulation of cell proliferation (Norman et al. 1989; du Cros et al. 1992). The cellular distribution of TGFα in sheep skin is consistent with inhibition of cell proliferation and/or promotion of differentiation, rather than simply with a mitogenic mode of action. Transforming growth factor-α has been implicated in fibre growth, and although many studies have demonstrated the mitogenic activity of TGFα, there is evidence of an opposite effect on certain cell types in skin, particularly within fibre follicles. In wool follicles, TGFα was localised
within the innermost cells of the outer root sheath and in cortical cells in the keratogenous zone, but absent from inner root sheath or dermal papilla cells (Nixon et al. 1995b). Sebaceous and sweat glands have high levels of this growth factor (Nixon et al. 1994b). Transforming growth factor-α has been shown to retard hair growth in mice (Wynn et al. 1989). However, in follicle culture, cell proliferation is stimulated in the presence of TGFα (Weinberg et al. 1990).

Like EGF, TGFα stimulates the secretion of collagenolytic enzymes by inducing the processing of collagenases which degrade type I collagen. Their regulation may be important in down-growth of the developing hair follicle through the dermis during initiation of the anagen growth phase (Weinberg et al. 1990). The potential to secrete collagenases in response to growth factors might also be important in cutaneous wound healing.

2.9.6 GROWTH HORMONE

Growth hormone is secreted by the pituitary in a pulsatile manner under the regulation of two hypothalamic peptides, growth hormone releasing hormone and somatostatin, which are stimulatory and inhibitory respectively (Fletcher et al. 1992; Magnan et al. 1995). The secretion of growth hormone is influenced by various conditions including stress and nutrition (Magnan et al. 1995). Typically the release of growth hormone induces an increase in the circulating concentration of insulin-like growth factor-I (Hua et al. 1993). The effects of exogenous growth hormone supplementation on wool growth have been equivocal; both increases and decreases in wool production have been reported (Johnsson et al. 1985; Wynn et al. 1988).

2.9.7 GLUCOCORTICOIDS

The glucocorticoids (hydrocortisone and cortisol) are released from the adrenal gland in response to stress induced release of adrenocorticotropic hormone from the pituitary (Wallace 1979). Glucocorticoids appear to have direct effects on wool protein synthesis and have been shown to variously inhibit or stimulate wool growth (Downes and Wallace 1965; Chapman et al. 1982; Moore 1982). Wool growth responses to environmental stress have also been reported to be both stimulatory or inhibitory depending on the duration and severity. These changes have been correlated with changes in plasma cortisol concentrations (Ferguson et al. 1965). It may be that stress induced wool breaks are due to an upregulation of epidermal growth factor (EGF) and the EGF receptor in the wool follicle, by cortisol (Behrendt et al. 1993a). In sheep, cortisol administration results in the loss of high affinity EGF binding in some animals (Behrendt et al. 1993b). Other possible mechanisms of action of the glucocorticoids include regulation of the immune system via major histocompatibility complex antigen expression (Breidahl et al. 1989), and interaction with cell membrane lipids (Epstein 1983). Receptor mediated activities include increased protein catabolism and decreased glucose uptake (Wallace 1979).

2.9.8 ETHANOLAMINE AND PHOSPHOETHANOLAMINE

Removal of the pituitary gland from sheep (hypophysectomy) stops wool growth in vivo after 4 to 26 weeks, with follicles entering telogen (Ferguson et al. 1965; Wallace 1979). In addition,
pituitary extracts have been shown to stimulate wool production both in vitro (Obana and Ito 1993) and in vivo (Ferguson et al. 1965; Wallace 1979). The pituitary extract examined was free of fibroblast growth factor (Obana and Ito 1993), growth hormone and thyrotrophic activity (Ferguson et al. 1965). One active component has since been identified as phosphoethanolamine, following the growth promoting activities of this growth factor on rat tumour cell lines (Tsao et al. 1982). This factor, in concert with ethanolamine, has been reported to have potent stimulatory effects on human keratinocyte growth (Hawley-Nelson et al. 1980; Breidahl et al. 1989).

2.10 Wool Follicle Culture

In vitro systems provide a unique opportunity to study and manipulate cellular processes outside the complex and frequently confounding influences of the in vivo situation. Information obtained using these growth conditions can then be used to examine whether similar processes occur in vivo. The genes involved in these processes can then potentially be identified and transgenic sheep created using recombinant DNA technology to establish germ line transmission (Seamark 1988; D'Andrea et al. 1988). Any efficient production of transgenic sheep which express the genes for wool fibre traits deemed desirable by fibre and textile consumers, is likely to greatly enhance the value of wool.

There is often a marked difference in the response of cells to a particular stimulus when they are cultured by different techniques (Norman et al. 1989). The choice of a particular in vitro system is largely determined by the experimental objectives. In order to identify the effects of growth factors on wool growth and fibre characteristics, it is necessary to differentiate between the three major methods of in vitro trichocyte maintenance; cell, explant and organ culture, and to determine which method has the most potential for elucidating the mechanisms of wool fibre growth.

2.10.1 Follicle Cell Culture

Keratinocyte culture systems consist of a disaggregated suspension of individual cells. Once the cells settle to the bottom of the flask they grow into a monolayer and become confluent (Breidahl et al. 1989). This in vitro approach has proven to be very useful for a number of systems (Hennings et al. 1979; Hawley-Nelson et al. 1980; Weterings et al. 1982; Thompson et al. 1985; Yuspa et al. 1988; Limat et al. 1991), however it suffers from two major disadvantages. Firstly, crucial cell-to-cell interactions are often disrupted and secondly, not all tissues are conducive to viable cell culture.

Both of these considerations apply with respect to trichocyte culture. Although some success is apparent in viable bulb cell isolation and maintenance (Frater and Hewish 1980), the cells are often short-lived, do not represent the whole bulb cell population, and do not synthesise cortical keratins (Frater and Hewish 1981). In addition, the disruption of intercellular signalling, particularly between dermal papilla and bulb cells is likely to significantly alter the activities and
differentiation of cultured trichocytes (Hynd 1989b). Dermal papilla cells maintained in vitro undergo changes in morphology and matrix synthesis, gaining features of normal fibroblasts and losing their specialised phenotype (Couchman et al. 1991). The initial formation of papilla-like clumps suggests that they retain some of their in vivo properties (Messenger 1984).

2.10.2 Follicle explant culture
Explant keratinocyte culture involves plating small blocks of skin onto a culture surface, and covering these with nutrient medium. Keratinocytes grow out from the edges of these skin blocks which contain intact wool follicles (Hardy 1951; Thompson et al. 1985; Breidahl et al. 1989; Holbrook and Minami 1991; Lingna et al. 1991). Skin explants closely resemble the structure of the original tissue. Unfortunately they have a number of disadvantages for follicle and fibre study. Cells, in addition to those of the follicle, are present. Consequently, paracrine factors from other dermal tissue may influence wool fibre synthesis (Breidahl et al. 1989). In addition, it is not possible to undertake detailed biochemical or morphological analysis of individual wool follicles (Philpott et al. 1989; Philpott et al. 1991). The size of the tissue section also gives rise to difficulties in nutrient transfer to much of the tissue. A further difficulty is that the ability of skin explants to grow a fibre is only identified in embryonic tissues. Following birth, no in vitro fibre growth has been successful using this technique (Frater and Whitmore 1973; Philpott et al. 1991).

2.10.3 Follicle organ culture
In recent years a number of researchers have successfully isolated intact hair follicles and maintained these in vitro with concomitant fibre production (Philpott et al. 1990). Kondo et al. (1990) described the organ culture of human scalp hair follicles and the effects of sex hormones on hair growth in vitro. Philpott et al. (1990) demonstrated that human hair maintained in organ culture grew at a rate which approximated in vivo hair growth. The increase in length was shown not to be associated with any disruption in hair follicle structure but was due to the formation of a keratinized hair fibre (Harmon and Nevins 1991). Hynd et al (1992) briefly examined this technique using sheep wool and rat vibrissa follicles and determined that in cultured wool follicles, production of a histologically normal fibre was occurring for up to seven days.

The method of Philpott et al. (1990) was less appropriate for rat vibrissa follicles for reasons that were not apparent (Hynd et al. 1992). Philpott et al (1989) has reported successful maintenance of newborn rat fibre follicles for seven days but they did note that all rat follicles displayed a premature entry into catagen under their conditions. Successful mouse pelage follicle culture has been described for up to eight days (Frater and Whitmore 1973; Frater 1980; Weinberg et al. 1990). When these follicles were reisolated from culture after 7 days they could successfully establish haired skin if implanted in the dermis of an immunologically incompetent hairless mouse (Weinberg et al. 1990). This implies that the morphology and activity of follicles is retained in organ culture.
Although isolated fibre follicles generally have a limited life span in vitro, because of the limitations of the in vitro system and because normal diploid cells have a finite life span (Norman et al. 1989), it seems likely that follicle organ culture is the most appropriate technique for the examination of factors regulating the control of wool fibre production. The method of Philpott et al. (1990) was used for all experiments described in this thesis on the basis of cost and simplicity.
No human investigation can be called true science without passing through mathematical tests.

Leonardo da Vinci (1452-1519)
3 MATERIALS AND METHODS

3.1 Wool Follicle Isolation
   3.1.1 Experimental procedure
   3.1.2 Results and discussion

3.2 Wool Follicle Culture
   3.2.1 Nutrient medium
      3.2.1.1 Preparation of the base medium
      3.2.1.2 Preparation of culture medium supplements
      3.2.1.3 Preparation of the control medium
   3.2.2 The in vitro culture environment

3.3 Measurement of Wool Fibre Growth
   3.3.1 Analysis by regression
   3.3.2 Fixed time analysis
   3.3.3 Conclusions about determining the rate of wool fibre growth

3.4 Statistical Treatment of Data
   3.4.1 Distribution of the rate of fibre growth by follicles maintained in vitro
   3.4.2 Chi squared analysis of follicle viability in vitro
      3.4.2.1 \( \chi^2 \) analysis of data to evaluate differences in follicle viability at two temperatures
3  MATERIALS AND METHODS

The information presented here outlines materials and methods common to all experiments in this study. Procedures specific to individual experiments are detailed in the appropriate experimental chapter.

3.1  WOOL FOLLICLE ISOLATION

Individual wool follicles were isolated from skin biopsies collected from the mid-side of adult sheep housed outdoors at pasture at a location 43°38'S. The midside of a selected animal was shorn and the skin sterilised by washing with 70% ethanol. Alcohol contamination of the sample was avoided by rinsing the area with sterile saline and drying the skin with sterile swabs. Approximately 0.5ml of local anaesthetic (Lignovet™; 20% w/v lignocaine hydrochloride; C-Vet Ltd, UK) was injected intradermally into the area from which the sample was to be collected.

During preliminary work it was found that the ease of follicle isolation was improved when narrow skin strips rather than large skin biopsies were collected. For all experiments described in this study, individual follicles were isolated from skin strips (0.5mm x 30mm). The necessity of collecting these very narrow skin strips required modification of a surgical scalpel handle so that two scalpel blades separated by a thin sheet of copper foil could be attached. Plate 3-1 shows the use of this modified surgical instrument, and Plate 3-2 shows a Nile blue sulphate stained skin section (Maddocks and Jackson 1988) collected by this method. In the stained section, wool follicles appear dark blue with connective tissue and wool fibres unstained. This staining procedure is applied here only to clarify detail and was never used prior to follicle dissection for tissue culture. Individual follicles are clearly visible in these narrow skin strips, making them reasonably easy to isolate.

Plate 3-1  Photograph demonstrating the method for collecting skin strip biopsies using the modified scalpel apparatus.
The appearance of follicles in skin strips collected with the double-bladed scalpel. Nile blue sulphate staining (Maddocks and Jackson 1988) was applied here to clarify detail (20X magnification). Wool follicles appear dark blue with connective tissue and wool fibres remaining unstained.

Following collection of the skin strip biopsy, individual follicles were isolated for maintenance in tissue culture. In the literature, four techniques for the isolation of fibre follicles have been reported; plucking, shear isolation, collagenase digestion and microdissection. Each of these techniques is summarised here and an experimental evaluation of two techniques, collagenase digestion and microdissection, is presented.

Plucking involves the extraction of fibres from intact skin using a pair of tweezers. Follicles isolated by this technique often do not maintain an intact dermal papilla (Weterings et al. 1982), and exhibit many fractures at the follicle bulb with resultant cellular disruption to the outer root sheath and connective tissue layer (Green et al. 1986). As a consequence, follicle morphology is altered considerably by this technique.

Large quantities of intact follicles can be isolated from biopsy samples using shear isolation. This technique involves the repeated cutting of skin with a loosely fitting pair of scissors (Green et al. 1986). Philpott et al. (1989) demonstrated that morphological disruption of the follicle occurred by this method of isolation which resulted in premature entry of the follicles into a catagen-like state, probably due to the effect of isolation stress. Follicles isolated by this technique appear unsuitable for the development of an in vitro model of wool growth.

The use of collagenase to isolate mouse fibre follicles has been described by Rogers et al. (1987). Keratin synthesis was frequently found to be disrupted and follicle morphology altered in follicles isolated by this technique. This is probably due to damage to the collagen sheath by the collagenase enzyme used for isolating the follicles. The advantage of collagenase is that it enables very rapid isolation of the follicles compared to other techniques.
Microdissection of fibre follicles from the skin involves the isolation of individual follicles from surrounding tissues under a binocular dissecting microscope using very fine needles (Messenger 1984). Follicle yield per unit time is very low but intact follicles with no visible structural damage are obtained. Fibre follicles isolated by this technique, and maintained in vitro, continue to produce a viable fibre (Frater and Hewish 1980; Green et al. 1986; Philpott et al. 1990; Kondo et al. 1990).

The suitability of collagenase digestion and microdissection for the isolation of wool follicles are compared below.

3.1.1 EXPERIMENTAL PROCEDURE

The effect of collagenase digestion on isolated wool follicles was examined by placing skin biopsies in 0.35% collagenase (Type V, Sigma Chem. Co. Mo USA) at 37°C for one hour prior to isolation of individual follicles by grasping each follicle near the skin surface with a pair of fine tweezers and gently pulling from the connective tissue. Follicles were subsequently maintained in tissue culture as described below (Section 3.2).

Additional skin strip biopsies were placed in a petri dish with approximately 10ml Control Medium A (defined in Section 3.2.1) without the addition of collagenase and maintained at 37°C for one hour. Individual follicles were isolated from the biopsy tissue using a binocular dissecting microscope to view the follicles, and very fine needles (Micro-Fine IV, U-100 insulin syringe needles) to dissect out individual follicles free of surrounding tissues (Messenger 1984).

3.1.2 RESULTS AND DISCUSSION

The technique by which follicles were isolated from skin biopsies affected the in vitro survival rate of follicles. Following collagenase digestion 62% of the isolated follicles had in vitro growth rates less than 30μm per day, indicating they failed to survive the isolation and culture procedure. In contrast, 92% of the follicles isolated by microdissection were viable. Chi squared analysis showed this difference was significant (P < 0.001). As shown in Table 3-1, there was also a significantly lower fibre growth rate by collagenase treated follicles compared with microdissected follicles (P < 0.001 by analysis of variance, Section 3.4). Collagenase treated follicles also produced wool fibre for approximately half the duration of microdissected follicles (2.5±0.1 days and 4.3±0.2 days respectively; mean ± S.E.M.), a difference that was highly significant (P < 0.001 by analysis of variance). Wool growth rate and follicle viability data provided in Table 3-1 includes only those follicles which produced fibre at a rate greater than 30μm per day.
In conclusion, follicles isolated by microdissection produce a wool fibre that grows significantly faster and survives in culture for a longer period of time than follicles isolated by collagenase digestion. Microdissection was therefore selected as the optimal technique for isolating viable wool follicles and was used for all experiments in the current study.

A further group of follicles was isolated by an adaptation of the microdissection technique by cutting them at the sebaceous gland level in an attempt to ease their removal from dermal connective tissue. This procedure was found to have no effect on the in vitro rate of wool growth (Table 3-2).

For the experiments described in this study, follicles were generally isolated in their entirety. In a few instances when the dermal layer was particularly tough, follicles were cut at the sebaceous gland prior to isolation. In some instances, the skin strip included a number of follicles which had been cut along the follicle shaft during biopsy collection. These follicles were not excluded from experiments if reasonably complete follicles could be isolated.

### 3.2 Wool Follicle Culture

Isolated follicles were maintained in 500μl of nutrient medium in a humidity and temperature controlled environment. Initially the culture conditions described by Philpott et al. (1989) for the maintenance of human hair in vitro were emulated, and are detailed below. In Chapter 5 of this study, the effect of modifying this culture methodology is examined with the aim of developing a system specifically optimal for the growth of wool fibres in a culture medium.
3.2.1 NUTRIENT MEDIUM

The nutrient medium used initially was termed Control Medium A. It is a supplemented, serum-free nutrient medium previously described by Philpott et al. (1989). Control Medium A is defined as William's Medium E supplemented with L-glutamine (1 mM), insulin (10 μg/ml), transferrin (10 μg/ml), sodium selenite (10 ng/ml), hydrocortisone (10 ng/ml), [Sigma Chemical Co.], trace elements, Fungizone® (2.5 μg/ml) and penicillin/streptomycin (100 units/ml), [Gibco BRL]. Details of the preparation of Control Medium A are provided below.

3.2.1.1 PREPARATION OF THE BASE MEDIUM

The base nutrient medium was William's Medium E (W-4125; Sigma Chemical Co.). This was prepared by dissolving the lyophilised William's Medium E powder in 1L ddH₂O prior to the addition of 2.2g NaHCO₃. The pH was adjusted to pH 7.0 prior to filter sterilisation (0.2μm pore size). The base medium was then aseptically dispensed into sterile containers and stored in 100ml aliquots at 4°C until use. The maximum storage period was 30 days.

3.2.1.2 PREPARATION OF CULTURE MEDIUM SUPPLEMENTS

Stock solutions of some media supplements were prepared and stored at -20°C until use. Details of the preparation techniques for these are provided in Table 3-3.

<table>
<thead>
<tr>
<th>Component/ Catalogue No</th>
<th>Stock Solution Concentration</th>
<th>Final Concentration in Williams Medium E</th>
<th>Additional Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin/ I-1882</td>
<td>5 mg/ml</td>
<td>10 μg/ml</td>
<td>pH adjusted to facilitate solubilization</td>
</tr>
<tr>
<td>Hydrocortisone/ H-0135</td>
<td>5 μg/ml</td>
<td>10 ng/ml</td>
<td>1 ml 70% ethanol added prior to addition of d₄H₂O</td>
</tr>
<tr>
<td>Transferrin/ T-1283</td>
<td>5 mg/ml</td>
<td>10 μg/ml</td>
<td></td>
</tr>
<tr>
<td>Sodium selenite/ S-9133</td>
<td>20 μg/ml</td>
<td>10 ng/ml</td>
<td></td>
</tr>
</tbody>
</table>

Table 3-3 Details of supplements for which stock solutions were prepared.

The culture medium was also supplemented with trace elements, Fungizone® and a mixture of penicillin and streptomycin. Details of the preparation of these are presented below.

The trace element mix was provided by Gibco BRL (13014-014). This was presented as a lyophilised powder and contained, MnSO₄·H₂O (0.0169 mg/l), Na₂SiO₃·9H₂O (14.2 mg/l), (NH₄)₆M₀₇O₂₄·4H₂O (0.0124 mg/l), NH₄VO₃ (0.0585 mg/l), NiCl₂·6H₂O (0.0119 mg/l), SnCl₂·9H₂O (0.0113 mg/l). The trace element mix was rehydrated to 10 ml with sterile d₄H₂O then supplemented into the culture medium at the rate of 1 ml per 100 ml William's Medium E.

250 μg Fungizone® (15295-017, Gibco BRL) was rehydrated with 20 ml sterile d₄H₂O to 12.5 μg/ml. This was supplemented into the culture media at the rate of 2.5 μg/ml to control fungi and yeasts.
1 ml of a solution containing 10,000 units/ml penicillin G and 10,000 µg/ml streptomycin sulfate (15140-031, Gibco BRL) was added to 100 ml William’s Medium E to control gram-positive and gram-negative bacteria.

3.2.1.3 Preparation of the control medium

Control Medium A was prepared by supplementing an aliquot of William’s Medium E with insulin, hydrocortisone, transferrin, sodium selenite, trace elements, Fungizone® and penicillin/streptomycin. The solution was filter sterilised (0.2 µm pore size) into an autoclaved Schott™ bottle in a biohazard cabinet and stored at 4°C for a maximum of seven days prior to use.

During the course of the research described in the current study, the medium was modified from Control Medium A to Control Medium B for reasons discussed in Chapter 5. Control Medium B was prepared by supplementing an aliquot of William’s Medium E with only insulin, hydrocortisone, transferrin and sodium selenite. This solution was also filter sterilised (0.2 µm pore size) into an autoclaved Schott™ bottle in a biohazard cabinet and stored at 4°C for a maximum of seven days.

3.2.2 The in vitro culture environment

The pH, temperature and atmosphere used for the in vitro maintenance of wool follicles were according to Philpott et al. (1989). Unless specifically indicated, follicles were maintained at 37°C in a humidified atmosphere of 5% CO₂ / 95% air. The pH of the culture medium was adjusted to pH 7.0-7.2 prior to filter sterilisation. This pH range has been defined as optimal for the culture of epidermal cells (Breidahl et al. 1989).

3.3 Measurement of wool fibre growth

In order to provide a useful model of wool growth, fibre production in vitro must occur at a measurable rate for a reasonable period. Philpott et al. (1989) showed that human hair follicles maintained in tissue culture grew at approximately 300 µm per day for four days. They did not define whether this was the maximum growth duration for these follicles. It appears a reasonable hypothesis that wool follicles maintained in vitro would also produce fibre at a consistent rate for at least four days.

For the work presented in this current study, wool fibre length was determined by measuring the distance from the end of the emerging wool fibre to the base of the follicle bulb from which it was generated. This measurement was undertaken by image analysis (Bioquant IV, R & M Biometrics, USA) at 144X magnification. Two techniques were evaluated to identify the optimum method for determining fibre growth rate. These were termed analysis by regression and fixed time analysis and are discussed in detail in the following two sections.
3.3.1 Analysis by regression

Wool follicles were dissected from the skin of one Romney sheep and maintained in Control Medium A. The length of each follicle plus fibre was measured every 24 hours and plotted against time (Figure 3-1). Only follicles which were deemed viable by demonstrating an increase in overall length greater than 30μm per day were included in the analyses described here and in Section 3.3.2.

Longevity was estimated for each follicle by identifying the maximum length obtained by the fibre plus follicle (Lmax). Any error caused by slight day to day variation in fibre length was reduced by subtracting 100μm from this Lmax value to give the variable Lmax-100. Follicle longevity was then defined as the first day on which fibre length exceeded Lmax-100. Figure 3-1 further illustrates the method by which longevity was determined. In this example, the maximum fibre plus follicle length achieved (Lmax) was 3540μm. Lmax-100 for this follicle is therefore 3440μm. The follicle plus fibre length exceeded this Lmax-100 value after being maintained in culture for eight days, therefore eight days was defined as the longevity for this follicle.

![Figure 3-1](image)

**Figure 3-1** Method for determination of follicle longevity in culture. Day eight is the first day on which the fibre length exceeded Lmax-100, therefore this follicle had a longevity of eight days. The regression trendline for data up to day eight inclusive is shown (- - -).

Using the technique of analysis by regression, the slope of the regression trendline up to day eight (the follicle longevity) was used to determine length growth rate per day. For this follicle the wool growth rate was 460μm per day.

In order for the analysis by regression technique to be deemed suitable for evaluating wool growth rate, follicle plus fibre length must increase at a consistent rate over the lifetime of the follicle. To examine this hypothesis, follicle plus fibre length data were plotted against time for
40 follicles isolated from a Romney sheep (Figure 3-2). Data for follicles with the same longevity were averaged for clarity.

![Graph showing follicle longevity](image)

**Figure 3-2** Changes in follicle plus fibre length over time for follicles maintained *in vitro* (n = 40). Follicle longevity is also shown. Error bars indicate ± S.E.M.

On average, these follicles remained viable in culture for $7.6 \pm 0.2$ days (mean ± S.E.M.) with some follicles remaining viable for 11 days and producing 3mm of wool fibre over this period. Average wool growth rate for the forty follicles using analysis by regression was $228 \pm 11 \mu m$ per day. From Figure 3-2 it is apparent that follicle plus fibre length increases at a constant rate over the lifetime of the follicle. All follicles displayed a consistent rate of growth for at least four days. These observations indicate that analysis by regression is appropriate for the measurement of wool growth *in vitro*.

A second method for the calculation of fibre growth rate is fixed time analysis which involves measuring fibre plus follicle length on only two occasions, immediately following isolation, and again following the lapse of a predetermined period of time in tissue culture. This technique is evaluated below.

### 3.3.2 Fixed time analysis

Data provided in Figure 3-2 showed that follicles isolated from a Romney sheep continued to increase in length at a constant rate for at least three days. Evaluation using the fixed time analysis technique was performed using the data for these follicles provided in Section 3.3.1.
Follicle plus fibre length at isolation and following three days maintenance in tissue culture was used to calculate the fibre growth rate by these follicles using the formula:

\[
\text{Fibre growth rate (\(\mu m/day\))} = \frac{\text{length at day three} - \text{length at isolation}}{3}
\]

While most fibres grew for more than three days, this time-frame was used to avoid any error due to fibres which displayed a reduced growth at about this time. The wool growth rate for the 40 isolated Romney follicles discussed previously, using fixed time analysis, was 290±14\(\mu m\) per day (mean ± S.E.M.). Follicle longevity could not be calculated using this technique.

**3.3.3 CONCLUSIONS ABOUT DETERMINING THE RATE OF WOOL FIBRE GROWTH**

Forty isolated wool follicles were maintained *in vitro* and the follicle plus fibre length determined daily. The calculated wool growth rate was significantly higher using the method of fixed time analysis (290±14\(\mu m\) per day) than by using analysis by regression (228±11\(\mu m\) per day). This difference was significant at \(P < 0.001\).

A modification of the analysis by regression technique was used to examine the validity of fixed time analysis. The slope of the fibre growth rate regression line for each follicle was determined to day three only, rather than for the lifetime of the individual follicle. The average wool growth rate for the 40 follicles, using this method, was 291±14\(\mu m\) per day. As this is identical to the value obtained using fixed time analysis (\(P = 0.96\)), it is apparent that the rate of fibre growth over the first three days is linear. This provides support for use of the fixed time analysis technique for determining the rate of fibre growth.

Fixed time analysis requires measurement of fibre plus follicle length on only two occasions (initially and after three days) in comparison with analysis by regression which requires measurement at 24 hour intervals. The former technique is therefore considerably less time consuming. The disadvantage of fixed time analysis is that follicle longevity *in vitro* is not determined. It is also possible that applied treatments may reduce longevity to less than three days. This would not be detected by fixed time analysis or would aberrantly be identified as a reduction in fibre growth rate.

Despite these potential disadvantages of fixed time analysis, this technique was used for the determination of wool growth rate for all experiments described in the current study due to the ease of measurement and potential increase in accuracy. Where longevity is reported, this was determined using the criteria of Lmax-100 described in Section 3.3.1.

**3.4 STATISTICAL TREATMENT OF DATA**

Replicate measurements of follicle length by image analysis indicate that the measurement of 30\(\mu m\) per day wool growth is close to the limit of measurement and experimental error possible with the equipment available. This compares with an average of approximately 200\(\mu m\) per day.
wool growth for most experiments presented in this study. Wool follicles with a fibre growth rate of less than $30 \mu m$ per day were therefore assumed to be non-viable and excluded from analysis of variance calculations in all experiments.

Calculation of the analysis of variance was undertaken to determine the probability that treatment and control follicle growth rates were drawn from populations with the same mean. Analysis of variance of viable follicles was undertaken using the statistical package GENSTAT 5 (Rothamsted Experimental Station, UK). Details of the analysis of variance program used for analysing data in this current study, and the information generated by this statistical technique is provided in Appendix A.

From preliminary data (Winder et al. 1992) it was found that there was a high degree of variation in the rate of wool growth between individual follicles isolated at the same time. It was determined that 20-30 replicate follicles are required in order for a difference of $50 \mu m$ per day to be significant at $P < 0.05$ using analysis of variance. Where possible, a sample size of at least 20 follicles has been used in order to enable statistical evaluation of treatment effects.

Analysis of variance of any data relies on the assumption that the data is normally distributed. In order to test this assumption, the distribution of the wool fibre growth rates by follicles maintained in vitro is evaluated here.

3.4.1 DISTRIBUTION OF THE RATE OF FIBRE GROWTH BY FOLLICLES MAINTAINED IN VITRO

Wool follicles were isolated from skin strip biopsies on a number of occasions from one English Leicester sheep. Follicles were maintained in vitro in Control Medium A and the frequency distribution of fibre growth rates determined ($n = 257$). As shown in Figure 3-3, the distribution of wool growth rate in vitro for follicles isolated from this sheep approximates that observed in normal sheep with an average growth rate of $321 \pm 10 \mu m$ per day (mean $\pm$ S.E.M.) and a range of $30-718 \mu m$ per day. This observation verifies that the assumption of a normal distribution is valid.
EXPERIMENTAL METHODS

3.4.2 CHI SQUARED ANALYSIS OF FOLLICLE VIABILITY IN VITRO

Some of the experiments evaluated here were aimed at inhibiting fibre growth. On these occasions, $\chi^2$ analysis was performed on all data (including wool growth rate values less than 30 μm per day) to determine whether applied treatments inhibited follicle viability in vitro. The following formula was applied to determine whether differences in the proportion of viable follicles between treatments was significant:

$$\chi^2 = \frac{(\text{observed proportion viable} - \text{expected proportion viable})^2}{\text{expected proportion viable}}$$

An example of the use of this statistical technique is provided here.

3.4.2.1 $\chi^2$ ANALYSIS OF DATA TO EVALUATE DIFFERENCES IN FOLLICLE VIABILITY AT TWO TEMPERATURES

<table>
<thead>
<tr>
<th>NUMBER OF FOLLICLES</th>
<th>CONTROL (37°C)</th>
<th>TREATMENT (31°C)</th>
<th>SUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>grew &gt; 30 μm/day</td>
<td>40</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>grew &lt; 30 μm/day (nogrow - NG)</td>
<td>3</td>
<td>31</td>
<td>34</td>
</tr>
<tr>
<td>TOTAL</td>
<td>43</td>
<td>41</td>
<td>84</td>
</tr>
</tbody>
</table>

Figure 3-3 The frequency distribution of the fibres growth rate by follicles isolated from an English Leicester sheep ($n = 257$).
expected overall proportion of nogrows (EP) = sum NG / sum Total
= 34 / 84
= 0.405

expected treatment NG (ENG) = Total (treatment) * EP
= 41 * 0.405
= 16.6

treatment $\chi^2$ = (NG (treatment) - ENG)$^2$ / ENG
= (31-16.6)$^2$ / 16.6
= 12.5

where 5% significance = 3.84; 1% significance = 6.64

The $\chi^2$ analysis described here provides evidence that maintenance of follicles at a temperature of 31°C rather than the control temperature of 37°C significantly reduced follicle viability ($P < 0.01$).
Results! Why man, I have gotten a lot of results. I know several thousand things that won’t work!

Thomas Edison
4 CHARACTERISATION OF IN VITRO FIBRE PRODUCTION

4.1 An Evaluation of In Vitro Follicle and Fibre Morphology
   4.1.1 Low magnification photomicrographs
   4.1.2 High magnification scanning electron micrographs
   4.1.3 Bromodeoxyuridine uptake by dividing cells
   4.1.4 Polymerase chain reaction amplification of keratin gene DNA
   4.1.5 Discussion

4.2 Evaluation of Fibre Growth in Nutrient Free Medium
   4.2.1 Experimental procedure
   4.2.2 Results and discussion

4.3 Correlation of In Vivo Wool Growth Rate with that Observed In Vitro
   4.3.1 Experimental procedure
   4.3.2 Results and discussion

4.4 Correlation of Follicle Bulb Size with In Vitro Fibre Growth Rate
   4.4.1 Experimental procedure
   4.4.2 Results and discussion

4.5 The Influence of Maintaining Wool Follicles In Vitro, on Fibre Medulla Formation
   4.5.1 Experimental procedure
   4.5.2 Results and discussion

4.6 Discussion and Conclusions
The overall aim of the work described in the current study is to examine effects of potential growth regulating factors on wool production, by follicles maintained in vitro. Any evaluation of these treatments requires an understanding of the characteristics of wool growth in tissue culture under standardised conditions. Thus, it is necessary to establish that isolated wool follicles maintained under the culture conditions developed in this work provide a reasonable model of in vivo wool growth. This necessitates an examination of the growth, morphology and cellular processes occurring within isolated, cultured follicles.

An evaluation of the rate of fibre growth by isolated follicles was provided in the previous chapter. It was shown that a wool fibre is produced by isolated follicles at a constant rate for at least four days and that the distribution of fibre growth rates approximated normal. In the work discussed here, these findings were extended by evaluating the characteristics of wool produced by isolated follicles in tissue culture. Five specific factors were examined:

1. the morphology of wool follicles maintained in vitro, and of the wool fibres produced during this procedure, were examined by light and electron microscopy, 5’-bromo-2’-deoxyuridine uptake and polymerase chain reaction amplification of keratin gene DNA;
2. the production of fibre by isolated follicles in vitro, in the absence of supplementary nutrients, was examined to verify that continued fibre growth is not due to processes already underway in the follicle in vivo;
3. the rate of wool growth in vitro, by isolated follicles, was compared with that observed in vivo;
4. an evaluation was undertaken of any relationship between follicle size, using the measurement of follicle bulb diameter, with fibre growth rate in vitro; and
5. the proportion of medulla in individual wool fibres was examined to identify any effects of in vitro maintenance on medulla formation.

Each of these areas of wool growth is discussed below in further detail.

### 4.1 An Evaluation of In Vitro Follicle and Fibre Morphology

To be of value as an in vitro model of wool growth, cellular components of both follicles and fibres must remain viable, grow and retain their normal structure for a reasonable length of time in tissue culture. It is anticipated that isolated follicles in culture will meet these requirements as cell to cell interactions are preserved because the follicle is a complete organ. Therefore in vivo cellular differentiation and wool fibre growth are likely to continue.

Evidence from the maintenance of other fibre types in vitro indicates that external fibre morphology is maintained in tissue culture. Philpott et al. (1991) evaluated human hair fibre growth over 96 hours by light microscopy and found that no disruption of hair follicle or fibre architecture occurred; the observed length increase was attributed to the production of a
keratinised hair shaft. In the study presented here, external fibre morphology was evaluated by light and scanning electron microscopy.

The internal structure and cell turnover of wool follicles maintained *in vitro* were examined in this study by identifying the cellular incorporation of the DNA marker 5'-bromo-2'-deoxyuridine (BrdU). In previous work with human hair follicles, Philpott *et al.* (1990) demonstrated by [methyl-$^3$H]thymidine autoradiography that *in vitro* maintenance of follicles does not disrupt trichocyte proliferation and migration. BrdU is a thymidine analogue which becomes incorporated into DNA during mitosis (Adelson *et al.* 1990). It provides a useful marker for identifying the site of proliferating cells.

A second technique used here to evaluate DNA morphology was the amplification of keratin genes using *in situ* application of the polymerase chain reaction. Individual follicles were fixed, embedded in paraffin wax and sectioned. Reagents incorporating polymerase enzymes, capable of catalysing DNA replication, and keratin gene primers, were secured to the tissue surface and amplification of the gene was undertaken using a thermal cycler. Incorporation of digoxygenin-UTP enabled the non-radioisotopic detection of amplified DNA. This technique has not been previously used for the *in situ* amplification of keratin DNA in isolated wool follicles.

Each of these techniques for the evaluation of follicle and fibre morphology *in vitro* are discussed below in detail.

### 4.1.1 Low Magnification Photomicrographs

Individual follicles were isolated from the midside of a Romney sheep and maintained in Control Medium A. Low magnification (40X) photographs of each follicle were taken immediately following isolation, and after three days maintenance in tissue culture. An examination of the photographs enabled an evaluation of whether measured length changes were due to the production of a wool fibre, or to altered follicle morphology. Photographs of a typical follicle before and after maintenance *in vitro* are presented below (Plates 4-1 and 4-2).

It can clearly be seen from these photographs that the increase in length observed when follicles are maintained in tissue culture is due to the production of a wool fibre. Plate 4-1 shows a freshly isolated wool follicle and Plate 4-2 shows the identical follicle after three days maintenance in tissue culture. In Plate 4-2, growth of the outer and inner root sheaths as well as the fibre is apparent. This has previously been observed during the *in vitro* maintenance of murine (Arase *et al.* 1991) and human (Philpott *et al.* 1991) fibre follicles.
In some instances, adhesion of the wool follicle to the bottom of the culture dish was observed. An example of this is shown in Plate 4-3. The adhesion appeared to be due to fibroblast, connective tissue or outer root sheath outgrowths. Arase et al. (1991) observed similar outgrowths when plucked murine fibre follicles were maintained in vitro and these were identified as outer root sheath cells. There was no apparent effect of this follicle adhesion on the rate of fibre growth. This phenomenon was not examined in detail in this study.
4.1.2 **HIGH MAGNIFICATION SCANNING ELECTRON MICROGRAPHS**

External wool follicle morphology was also examined by scanning electron microscopy. Isolated follicles from an English Leicester sheep were maintained in Control Medium A for seven days. Following this period, follicles which showed evidence of fibre growth under the light microscope were examined under the scanning electron microscope.

Approximately 2mm of fibre could typically be seen protruding from each follicle at the time of isolation from skin biopsies. The region around the fibre tip was assumed to be representative of wool growth *in vivo*. Furthermore, after seven days maintenance in tissue culture, the portion of fibre adjacent to the follicle was assumed to have been produced *in vitro*. This assumption is valid only if the total fibre length is greater than the initial fibre plus follicle length. Only follicles meeting this criteria were used for this study.

For analysis using the electron microscope, isolated follicles were retrieved from the culture medium after seven days and dehydrated in an ethanol gradient to 100% ethanol. Ethanol was then replaced by liquid CO$_2$ in a pressure vessel. The temperature (and hence the pressure) was gradually raised until the critical drying point was reached (31°C and 73 atmospheres for CO$_2$). Dried follicles were stored desiccated. Secondary electron detectors were used which necessitated that the specimens were electrically conducting, therefore a thin layer of gold was thermally evaporated onto each specimen. To achieve this, individual follicles were mounted onto metal stubs using colloidal carbon cement. Mounted specimens were coated with gold using an E5100 Coating Unit (Polaron, UK) at 20mA, 2.4kV. Coated follicles were stored desiccated until observed under the scanning electron microscope.
Mounted, gold-coated follicles were photographed at 650X and 1950X magnification following amplification using a Phillips CM12 scanning transmission electron microscope fitted with backscatter and secondary electron detectors.

The following two photographs show regions of a typical English Leicester wool fibre grown prior to follicle isolation (Plate 4-4) and during maintenance *in vitro* (Plate 4-5). These photographs show different portions of the same wool fibre at 1950X magnification.

Plate 4-4  Electron micrograph of a region of wool fibre produced by an English Leicester sheep prior to isolation of the wool follicle for maintenance *in vitro* (1950X magnification).

Plate 4-5  A region of the same wool fibre shown in Plate 4-4 which was synthesised during maintenance of the follicle *in vitro* (1950X magnification).
The photographs presented as Plates 4-4 and 4-5 provide additional evidence that the external morphology of wool fibres produced in vitro is comparable with that observed in vivo. A more quantitative evaluation was undertaken by measuring the distance between consecutive cuticle ridges on the surface of thirteen fibres, by image analysis. Cuticle ridge length at the fibre tip (deemed to have been produced in vivo) was compared with that occurring near the opening of the follicle (fibre produced in vitro). This study was undertaken at 650X magnification.

The results of this analysis are presented in Table 4-1 and verify previous observations which suggest that the external morphology of wool fibres grown in tissue culture does not differ from that produced in vivo.

<table>
<thead>
<tr>
<th>Fibre produced</th>
<th>number of cuticle ridges measured</th>
<th>fibre cuticle ridge length (650X magnification) mean ± S.E.M. (mm)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>before isolation</td>
<td>96</td>
<td>12.1±0.4</td>
<td></td>
</tr>
<tr>
<td>after in vitro culture</td>
<td>103</td>
<td>11.4±0.3</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Table 4-1 Distance between consecutive cuticle ridges on wool fibres from freshly isolated follicles and following maintenance in tissue culture.

There was no difference in the distance between adjacent cuticle ridges for portions of individual fibres synthesised in vivo and those produced in vitro (P > 0.01).

4.1.3 Bromodeoxyuridine uptake by dividing cells

Internal morphology following in vitro culture of isolated follicles was evaluated by identifying sites of 2'-bromo-5'-deoxyuridine (BrdU) uptake. This technique provides an estimate of cell birth, or mitotic rate, and has previously been used to evaluate cell proliferation in the wool follicle bulb (Hynd and Everett 1990).

Wool follicles isolated from a Romney sheep were maintained in Control Medium A for three days prior to incubation in Control Medium A containing 10µM BrdU for four hours. Follicles were removed from the BrdU supplemented medium, fixed in Bouin’s solution for two hours, dehydrated in ethanol and cleared in xylene prior to paraffin wax embedding. Follicles were sectioned at 6µm and the sections deparaffinized, hydrated and treated with 1.5M HCl for 30 minutes to denature the DNA. Sections were then washed with saline/Tween 20 (4°C) and incubated with anti-BrdU (Becton Dickinson reagent diluted 1:280 in phosphate-buffered saline/Tween 20/1%BSA) for 30 minutes. Following this antibody incubation, sections were washed and incubated with biotinylated horse-anti-mouse immunoglobulin for 30 minutes, then with avidin/biotin complex (Vectastain kit, Becton Labs, Burlingame, Ca., USA) for 60 minutes. Sections were washed in phosphate buffered saline followed by a 0.1% solution of diaminobenzidine tetrachlorohydrochloride activated with H₂O₂ for 15 minutes. Slides were washed and stained in Haematoxylin prior to image analysis, at 100X magnification, of follicle bulb sections to identify mitotic figures by the presence of amber pigment. The technique described here is a modification of that developed by de Fazio et al. (1987).
Evaluation of the sites of incorporation of BrdU in the follicle bulb showed that both rapidly growing and slow growing follicles displayed patterns of DNA synthesising cells which were identical to those observed in vivo (Adelson et al. 1991). BrdU incorporation into the bulb of a follicle which had been maintained in tissue culture for three days is shown in Plate 4-6. Cell mitosis is indicated as a dark pigment where BrdU has been incorporated into the DNA of dividing cells.

Plate 4-6  BrdU incorporation by cells of the wool follicle bulb following maintenance of the follicle in vitro for three days in Control Medium A. Follicles were then incubated in medium supplemented with BrdU for four hours. 100X magnification.

It can be seen from Plate 4-6 that cell proliferation is occurring primarily in the germinative region of the follicle bulb, with little proliferation occurring in the dermal papilla. This pattern of mitosis has been previously described in follicle bulbs in vivo (Adelson et al. 1990; Philpott et al. 1991; Ibraheem et al. 1993a). This evidence supports the hypothesis that cell division is occurring in isolated follicles which have been maintained in vitro for three days, and that it is occurring in a manner typical of that observed in vivo.

4.1.4  Polymerase chain reaction amplification of keratin gene DNA

A final technique used to evaluate the viability of isolated wool follicles was the location of intact nuclear deoxyribonucleic acid (DNA). Nuclear DNA is expected to be present in all normal cells as it is from this template that all cellular proteins are formed. Significant mutation or degradation of DNA can be observed by labelling DNA molecules in situ by polymerase chain reaction (PCR) amplification.

Examination of nuclear DNA was undertaken by isolating a number of wool follicles from the skin of an English Leicester sheep. One group of follicles was fixed in formal saline immediately upon isolation. A second group of follicles was maintained in Control Medium A for three days prior
to being fixed in formal saline. Following fixation, both groups of follicles were treated identically.

Full details of the PCR amplification procedure used in this study are provided in Appendix B. Briefly, follicles were embedded in paraffin wax and 5μm sections cut using a standard microtome. Sections were secured to the surface of poly-L-lysine coated microscope slides, deparaffinized with xylene and hydrated using an ethanol gradient. Tissue sections were treated with pepsin to reduce the degree of crosslinking produced by the fixative and to ensure membrane porosity. The optimum duration of protease digestion was determined experimentally for this study by treating sections with pepsin for between 0 and 60 minutes. Reactants for PCR must be able to enter the cell, but reaction products must be retained. Maintenance of tissue sections in pepsin for 30 minutes was found to be optimal in meeting these criteria. Following protease digestion, tissue sections were post-fixed in formal saline to improve the structural integrity of the tissue.

DNA amplification was undertaken using PCR. The method of Rogers et al. (1994), which was developed for DNA prepared from homogenised tissues, was adapted for in situ PCR amplification, and incorporated the modified nucleotide, digoxygenin-UTP. Twenty five microlitres of PCR reaction mix (Appendix B) was applied to each microscope slide with adherent tissue sections, and sealed in place with a coverslip. Thermal cycling was undertaken for 30 cycles to amplify the keratin gene coded for by the primers selected for this study. Full details of the primers selected are provided in Appendix B.

Following PCR amplification, the coverslips were removed and the slides washed in phosphate buffered saline. Amplified keratin gene sites were identified using the digoxygenin detection system (Boehringer Mannheim; detailed in Appendix B). Morphological tissue detail was clarified by Eosin-Y staining prior to permanent mounting of the tissue sections in DPX. Mounted sections were examined using a light microscope.

Examination of follicle sections treated using this technique showed that for both freshly isolated, and cultured wool follicles, up to 95% of nuclei were specifically labelled following digoxygenin incorporation. However the results were inconsistent. Consequently, positive and negative control sections were always run concurrently with test samples. In Plates 4-7 to 4-11, dark blue spots indicate the site of nuclear DNA amplification, where digoxygenin-UTP has become incorporated. The pink colouration of wool fibres in these plates is the result of Eosin-Y staining.

Plate 4-7 shows the PCR amplification of keratin gene DNA for a wool follicle which had been maintained in vitro for three days. Although some tissue disruption has occurred due to the rigorous experimental conditions, the follicle bulb, tissue root sheaths and wool fibre can clearly be differentiated. Intact nuclear DNA is evident in the germinal matrix, dermal papilla and fibre root sheath regions. Intact DNA appears to be reduced as the fibre becomes keratinised.
Polymerase chain reaction amplification of keratin gene DNA in a longitudinal section of an isolated wool follicle which had been maintained \textit{in vitro} for 72 hours. Dark spots indicate the site of gene amplification and incorporation of digoxygenin. Pink colouration is due to Eosin-y staining. 100X magnification.
In Plates 4-8 to 4-11, transverse sections of wool follicles which have not been maintained in vitro, are shown at 1000X magnification. Each plate shows an example of one of the four experimental controls evaluated.

Plate 4-8 is presented as a positive control. In this plate, the follicle section has undergone a procedure identical to that of the section presented in Plate 4-7. Dark blue spots in the follicle root sheaths indicate intact nuclear DNA. Pink staining of the fibre, with intact nuclei, can also be seen.

Plate 4-8 Transverse section through a wool follicle showing PCR amplification of keratin gene DNA (1000X magnification).

Plate 4-9 shows the effect of Eosin-Y staining. None of the protease digestion or gene amplification techniques were performed on this tissue section. From Plate 4-9 it is apparent that in the absence of PCR gene amplification, no dark blue nuclei occur.
Plate 4-9  Transverse section through a wool follicle which has been Eosin-y stained only, no gene amplification has been undertaken (1000X magnification).

The tissue section in Plate 4-10 has undergone protease digestion and PCR amplification however the DNA polymerase enzyme, Taq polymerase, was not included in the reaction mix. This slide is therefore a negative experimental control. From this slide it can be seen that digoxygenin incorporation, and therefore blue staining, occurs only as a result of DNA amplification by PCR and that there is no non-specific binding. When the enzyme required for DNA amplification is not present, blue stained nuclei do not occur. Pink colouration of the fibre and inner root sheath can be seen. This results from Eosin-Y staining.

Plate 4-10  Transverse section of a wool follicle showing the effect of excluding the enzyme Taq polymerase from the PCR reaction mixture. Pink colouration results from Eosin-Y staining. 1000X magnification.
The tissue section shown in Plate 4-11 provides confirmation that pepsin digestion, prior to PCR amplification, is essential. This section was not subjected to protease digestion and consequently, there has been no amplification of nuclear DNA during PCR.

Plate 4-11 Transverse section of a wool follicle which has not undergone protease digestion prior to keratin gene amplification by PCR.

In tissue sections which have undergone normal PCR amplification (Plate 4-7 and Plate 4-8), incorporation of the digoxigenin nucleotide by amplification of keratin gene DNA was apparent within the follicle bulb, the inner and outer root sheaths and associated connective tissue. As this technique does not identify the expression of keratin genes, but rather the existence of intact DNA, the sites of keratin gene localization are consistent with structurally intact DNA being present within all cells of the wool follicle after three days maintenance in vitro.

4.1.5 DISCUSSION

The microscopic evaluation of wool fibre structure presented here shows that there was no disruption of the fibre structure when follicles were isolated and maintained in vitro. Additionally, cell division in isolated wool follicles, as demonstrated by the uptake of BrdU, occurred in the germinative region of the follicle bulb and the outer root sheath. This concurs with previously described sites of cell division in vivo.

PCR amplification studies have also shown that the structural sequence of nuclear DNA is maintained in follicles grown in vitro. Amplification of keratin gene DNA occurred at equivalent sites in freshly isolated and cultured wool follicles. Loss of intact keratin DNA was observed in wool fibres at approximately the zone of keratinization. It has previously been reported that at this time, catabolic changes within wool fibres results in the lysis of cell organelles, their associated proteins and nucleic acids (Gillespie and Marshall 1988).
All these findings provide substantial evidence that the maintenance of wool follicles \textit{in vitro} results in the production of a wool fibre which is similar to that produced \textit{in vivo}.

### 4.2 Evaluation of Fibre Growth in Nutrient Free Medium

It has previously been shown that isolated follicles maintained under \textit{in vitro} conditions produce a wool fibre for at least four days but seldom for more than seven days. The possibility exists that this wool growth may be a carryover of \textit{in vivo} activity. It is therefore necessary to verify that the fibre production emanates from a response to the nutrients in the culture medium, rather than processes inherent in the isolated follicle. This hypothesis was examined by maintaining isolated follicles in physiological saline and observing any effects on fibre production \textit{in vitro}.

#### 4.2.1 Experimental Procedure

Microdissected follicles from the skin of one English Leicester sheep were maintained individually in 500\(\mu\)l of physiological saline. A second group of follicles from the same animal were maintained in Control Medium A. The growth rate of fibres from each group of follicles was determined over a three day period. The average longevity of the two groups of follicles was also determined.

#### 4.2.2 Results and Discussion

The average fibre growth rate was markedly reduced when follicles were maintained in saline. Table 4-2 shows that fibre production by viable follicles (length increase greater than 30\(\mu\)m per day) in physiological saline was only about 25\% of that recorded for follicles in Control Medium A. The difference was highly significant (\(P<0.001\)). There was also a significant reduction in the longevity of follicles \textit{in vitro} (\(P<0.001\)). The group maintained in physiological saline produced fibre for only 1.7±0.3 days (mean ± S.E.M.) in comparison to 3.1±0.6 days for follicles maintained in Control Medium A.

In addition, \(\chi^2\) analysis of all follicles maintained \textit{in vitro} showed that there was a significant (\(P<0.001\)) increase in the proportion of non-viable follicles when maintained in physiological saline. 27\% of follicles maintained in saline remained viable, compared with 96\% of follicles maintained in Control Medium A.

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± S.E.M., (\mu)m/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Medium A</td>
<td>24</td>
<td>23</td>
<td>165±22</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>physiological saline</td>
<td>22</td>
<td>6</td>
<td>44±2</td>
<td></td>
</tr>
</tbody>
</table>

Table 4-2 Effect of saline or Control Medium A on fibre growth in culture. The difference in the proportion of follicles which remained viable between treatments was significant by \(\chi^2\) analysis (** P<0.001).
This study shows that follicles isolated from the skin do not continue to produce a wool fibre when maintained in physiological saline. The saline solution provides an osmolarity equivalent to Control Medium A but has no nutrient components. This solution is not sufficient for the maintenance of wool follicles in vitro, suggesting that fibre production from follicles maintained in a nutrient medium is not due to an inherent follicle process already underway in the follicle, but to a response of the follicle to the culture conditions.

4.3 **CORRELATION OF *IN VIVO* WOOL GROWTH RATE WITH THAT OBSERVED *IN VITRO***

For the development of a useful tissue culture model of wool growth it was deemed useful to determine the correlation of wool growth in vitro with that occurring in vivo. This analysis was undertaken in this chapter.

The animals selected for this study were one English Leicester and one Drysdale sheep. Animals from these breeds have been documented to have annual wool growth rates in vivo of 150-200mm and 200-300mm per year respectively (Ross 1990) which equate to approximately 480\(\mu\)m and 680\(\mu\)m per day respectively. Both sheep breeds display seasonal fluctuations in fibre growth with the average growth rate per day in summer being higher than in winter. This observation must be borne in mind when comparing in vitro wool growth rates, which determines fibre growth at one point in time, with in vivo wool growth where wool production over a period of time is evaluated.

4.3.1 **EXPERIMENTAL PROCEDURE**

The in vivo wool growth rate by one English Leicester and one Drysdale sheep was determined by measuring the average staple length at April 1994 for wool which had been produced during the previous six months. This was achieved by randomly selecting animals which had been shorn in October 1993, and using a ruler to measure the distance from the skin of the animal to the tip of individual staples six months later (April 1994). The length of twenty staples from the midside of each of the two sheep were measured by this method.

In April 1994, individual wool follicles were isolated from the same animals and maintained in tissue culture. The rate of in vivo wool growth was then compared with the in vitro follicle fibre growth rate.

4.3.2 **RESULTS AND DISCUSSION**

The rate of in vivo wool growth by the English Leicester sheep was 129mm for the six month period which equates to a daily growth rate of 690\(\mu\)m. This exceeds the average fibre growth rate of 480\(\mu\)m per day estimated by Ross (1990). Wool growth of 1332\(\mu\)m per day was recorded for the Drysdale animal, which was approximately twice that of the English Leicester, and exceeds the growth rate of 680\(\mu\)m per day previously recorded by Ross (1990).
These apparently high wool growth rate data may reflect the timing of the study. That is, the present study was carried out over spring and summer which is a time when the rate of wool production is higher than the rest of the year (Bigham et al. 1978; Geenty et al. 1984).

Fibre growth by isolated follicles was $252 \pm 30 \mu m$ per day and $388 \pm 43 \mu m$ per day for the English Leicester and Drysdale sheep respectively (Table 4-3).

<table>
<thead>
<tr>
<th>Sheep breed</th>
<th>fibre growth rate (mean ± S.E.M.) ((\mu m) per day)</th>
<th>comparison of wool growth rates in vitro: in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>English Leicester</td>
<td>252±30</td>
<td>690±12</td>
</tr>
<tr>
<td>Drysdale</td>
<td>388±43</td>
<td>1332±18</td>
</tr>
</tbody>
</table>

Table 4-3 The rate of wool production in vivo and in vitro by one English Leicester and one Drysdale sheep. A comparison of wool growth under these two conditions is provided.

Thus the in vivo wool growth for each breed was approximately three times the rate of fibre growth in follicles isolated and maintained in vitro. This ratio was consistent in spite of the large difference in in vivo and in vitro wool growth rates between breeds.

It was noted that the rate of in vivo wool growth exceeded the average due to this study being conducted over spring and summer, a period of fast wool growth. In contrast, follicles for in vitro culture were isolated in Autumn, when wool growth is reduced (Geenty et al. 1984; Lincoln 1990). Therefore the observation that wool growth by isolated follicles occurs at one third the in vivo rate may not be accurate. What is interesting is that the ratio of in vivo to in vitro wool growth is consistent, and independent of sheep breed. However, it is necessary to examine a greater number of breeds to verify this observation. Furthermore, it would be valuable to examine whether the season in which the follicles are isolated has any effect on the rate of fibre production. Chapter 8 examines the effects of season on fibre growth, under in vitro conditions.

### 4.4 Correlation of Follicle Bulb Size with in Vitro Fibre Growth Rate

The morphology of the wool follicle has been discussed in detail in Chapter 2. In summary, the follicle bulb consists of a cellular matrix which surrounds the dermal papilla (Schinckel 1961), and a germinative or proliferative zone in the follicle bulb which extends to one cell layer above the apex of the papilla (Fraser 1965).

The rate of wool production in vivo appears to be highly correlated with the size of the wool follicle bulb and in particular with the number and size of cells in the germinative zone. Several workers have demonstrated that there is a strong positive correlation between the number of cells in the follicle bulb and, consequently, bulb diameter, with fibre diameter and the rate of fibre
Kelly et al. 1993). From these studies it appears that the rate of fibre output is largely dependent on the number of cells produced in the germinative region of the follicle bulb. A larger proliferative area in the bulb enables an increase in the number of migrating follicle cells which may potentially enter the fibre (Black 1987).

Given that larger follicles are capable of producing a greater volume of wool than smaller follicles, it seems a reasonable hypothesis that the in vitro rate of wool growth should also correlate with follicle size in isolated follicles maintained in culture. This hypothesis was evaluated in this chapter.

4.4.1 **Experimental Procedure**

Individual follicles (257) were isolated at random periods over twelve months from the midside of one English Leicester sheep. The diameter of each follicle bulb was measured at the widest point by image analysis. The follicles were then maintained in vitro for three days and the rate of fibre growth determined. The rate of wool growth was plotted against bulb diameter for each follicle.

4.4.2 **Results and Discussion**

Figure 4-1 shows the relationship between follicle bulb size and fibre growth rate in vitro.

![Figure 4-1](image_url)

**Relationship between follicle bulb size and fibre growth rate of follicles isolated from one English Leicester sheep and maintained in vitro.** Blue shapes indicate follicles with a fibre growth rate less than 550 μm per day; pink shapes indicate those which produced fibre at ≥ 550 μm per day.
There was a significant positive correlation between fibre growth rate \textit{in vitro} and follicle bulb diameter ($R^2=0.19; P<0.001$). The relationship between follicle bulb diameter and fibre growth rate is, however, best explained using a split trendline (Figure 4.1). This suggests that there are two independent components which account for the variation in this data. Follicles which produced a fibre at less than 550 $\mu$m per day had a low correlation with follicle bulb diameter ($R^2=0.05; P<0.001$), whereas bulb size was strongly correlated with wool growth in follicles where the growth rate was greater ($R^2=0.33; P<0.001$). This observations encourages speculations that the two components can be accounted for by primary and secondary follicle populations, and that these are regulated by discrete pathways. Indeed, the proportion of postulated primary and secondary follicles is comparable to that observed \textit{in vivo}. Differences in fibre growth cycles have been reported for the two follicle populations (Hardy 1992) and different initiation controls occur \textit{in utero} (Orwin 1988), therefore it is feasible that differential regulation occurs.

In their review, Black and Reis (1979) noted that the rate of fibre growth depends on the number and size of cells in the follicle bulb, and on the proportion of bulb cells which migrate and enter the fibre. The observation, in this study, that the rate of wool growth \textit{in vitro} is correlated with follicle bulb size, as occurs \textit{in vivo}, is therefore an important step in verifying that isolated follicles are a viable \textit{in vitro} model of wool growth. It indicates that there is no alteration in the commitment of cells from the germinative region, into the fibre, when follicles are isolated and maintained in tissue culture. This provides additional evidence that the follicle culture technique described in this thesis maintains viable follicles which are morphologically and physiologically similar to those operating under natural \textit{in vivo} conditions.

\section*{4.5 The Influence of Maintaining Wool Follicles \textit{In Vitro}, on Fibre Medulla Formation}

Fleece medullation is an important structural characteristic which affects the processing properties of wool fibres (Orwin 1979b). The medulla can be defined as a central core of cells in the fibre cortex which do not contain keratin proteins (Auber 1950) but appear to synthesise trichohyalin protein granules. During fibre development, the trichohyalin proteins form a hardened amorphous structure (Rogers 1959a; Orwin 1979a; Rothnagel and Rogers 1986). Production of a medulla appears to arise during the formation of wool fibres with a large volume (high length growth and/or diameter) where keratin protein synthesis is less than trichocyte production. The controlling factors regulating the formation of a medulla still need to be identified (Auber 1950).

Appreciable differences in the degree of medullation occur between breeds of sheep. Genetic, nutritional, seasonal and disease factors have each been implicated in affecting the degree of medullation (Auber 1950; Orwin 1979b; Chapman and Ward 1979; Scobie et al. 1993). As yet, quantitative details of breed and seasonal differences in medulla production have not been widely documented. This is primarily because techniques for measurement of the medulla remain reasonably subjective although a new method using optical fibre diameter analysis (OFDA) is currently under development (Justine Lee, pers.comm.).
Given that the presence of a medulla influences the processing qualities of wool, it is of interest to examine changes in medulla production in isolated wool follicles. Since in vitro wool growth appears to correlate with in vivo wool production, it would appear that medullated fibres will continue to produce a medulla when maintained in tissue culture. This hypothesis is examined in this chapter by comparing the amount of medulla in individual fibres before and after maintenance under in vitro culture conditions.

4.5.1 Experimental Procedure

Twenty wool follicles were isolated from the midside of one English Leicester sheep in midsummer (January 1993). Fibre plus follicle length was determined in follicles immediately after isolation and after maintenance in Control Medium A for seven days. Entire follicles were then mounted in a solution of benzyl alcohol and aniseed oil. This solution has the same refractive index as keratin protein which enabled regions of medulla and keratinised fibre to be clearly defined by phase contrast microscopy (Ross 1990).

A third fibre component with a refractive index different from that of keratin or medulla was often visible in the central cortical cells, the same location as the medulla. This component was termed 'pseudo-medulla' and was thought to be the medulla saturated with culture medium. Steps were taken to verify that this was indeed nutrient medium saturated medulla. For example, following initial analysis, fibres exhibiting pseudo-medulla were removed from the microscope slide, washed with ethanol and dried at 55°C for 36 hours prior to being re-mounted in benzyl alcohol:aniseed oil for further image analysis of fibre area, medulla area and fibre diameter.

Measurement of total cross-sectional fibre and medulla areas enabled determination of the proportion of medulla and pseudo-medulla in the fibre. The proportion of these components as a percentage of fibre area, as well as the fibre diameter, was determined at a number of locations along the fibre by image analysis of mounted follicles at 40X magnification. The initial follicle plus fibre length at isolation was used to identify portions of fibre produced in vivo and in vitro as shown diagrammatically in Figure 4-2 and as defined previously in Section 4.1.2. Only those follicles in which the fibre length exceeded the original fibre plus follicle length were used for this experiment.

![Figure 4-2](image-url)

**Figure 4-2**  Diagrammatic representation of follicle plus fibre following seven days maintenance in tissue culture. → → → shows the follicle length when initially isolated.
4.5.2 **Results and Discussion**

Twelve of the isolated English Leicester follicles continued to produce a wool fibre for seven days *in vitro*. For each of these follicles, the length of the fibre after tissue culture was greater than the original fibre plus follicle length. This confirmed that some of the wool fibre extending from the follicle was synthesised *in vitro*, as detailed in Figure 4-2.

In the portion of freshly mounted wool fibres produced *in vivo* the amount of medulla ranged from 0% to 48%. The proportion of an individual fibre which was pseudo-medulla ranged from 0% to 17%. The results of this study are presented in Table 4-4. The data show there was a significant difference in the average amount of medulla present in the two regions of fibre. While an average of 17.3% medullation was observed in the *in vivo* portion of fibres, a medulla was never present in the portion of fibre grown *in vitro*. Conversely, pseudo-medulla formed a very low proportion of the fibre grown before isolation (0.8%), and was more frequently observed in fibres produced *in vitro* (5.7%). There was no difference between the average diameter of the portion of fibre produced *in vivo* and *in vitro*.

When these fibres were dried for 36 hours, a different pattern of fibre morphology was observed. Pseudo-medulla was never apparent but appeared to be replaced with normal keratinised fibre in marked contrast with medullated regions which remained visible following drying of the fibre.

There was no significant increase in the proportion of medulla following drying, and a comparison of pre- and post-drying photographs showed that regions of pseudo-medulla were not converted to medulla upon drying of the fibre. The hypothesis that pseudo-medulla regions are fluid-filled medulla was not supported by these findings. It seems likely that pseudo-medulla is in fact keratinised cells with a slightly different composition and hence, the refractive index differs from other keratinised parts of the fibre. It was postulated by Scobie and Woods (1992) that there is likely to be a continuum of cortical cell types which differ in their protein compositions ranging from medulla, which contain no keratin protein, to paracortex which consist of very high sulphur keratin proteins. Pseudo-medulla is likely to be intermediate between these cell types.

Fibre diameter was also decreased by drying of the wool fibres. This outcome was expected given that keratin protein is hydrophilic and is normally associated with water molecules.

<table>
<thead>
<tr>
<th></th>
<th>medulla (%)</th>
<th>pseudo-medulla (%)</th>
<th>fibre diameter (microns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly mounted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>in vivo</em></td>
<td>17.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.8%</td>
<td>35.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>in vitro</em></td>
<td>0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.7%</td>
<td>35.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Following drying</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>in vivo</em></td>
<td>15.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0%</td>
<td>31.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>in vitro</em></td>
<td>0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0%</td>
<td>31.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 4-4 Details of the fibre diameter and proportion of medulla and pseudo-medulla in fibres grown by follicles isolated from an English Leicester sheep. The characteristics of the portions of fibre which had been produced prior to isolation (*in vivo*), and during tissue culture (*in vitro*) are compared. Column values which have the same superscript letter are significantly different (P < 0.001).
A medullated fibre was not produced \textit{in vitro} by isolated English Leicester follicles, even though the exact follicles produced a medullated fibre \textit{in vivo}. In contrast, preliminary observations have suggested that isolated wool follicles from Drysdale sheep do produce a medullated fibre \textit{in vitro}, albeit to a reduced degree. \textit{In vivo}, Drysdale fibres are highly medullated with up to 90\% of the fibre being occupied by a medulla (Ross 1990) compared to an average of 17\% medullation in the English Leicester fibres examined in this study. It seems likely that the occurrence of a medulla \textit{in vitro} in fibres from Drysdale sheep is not specifically a breed difference but rather a reflection of the greater level of medulla present in fibres of the Drysdale sheep. Two possible explanations for the loss or reduction of medulla by follicles maintained \textit{in vitro} are presented below.

Firstly it may be that there is a reduced volume of wool produced \textit{in vitro} relative to \textit{in vivo}. That is, whilst the fibre diameter was not reduced \textit{in vitro} (Table 4-4), there was a significant reduction in the rate of fibre length growth (Table 4-3). Hence, fibre volume must be reduced by \textit{in vitro} maintenance. Scobie and Woods (1992) suggested that medulla formation is due to the rate of production of fibre cells exceeding their ability to synthesise keratin proteins, due to limitations in the supply of sulphur amino acids. They hypothesise that as fibre volume increases, there may be insufficient keratin to form any type of cortical cell and therefore a medulla forms. Conversely, as fibre volume decreases, the occurrence of a medulla is reduced. During the maintenance of follicles \textit{in vitro}, the reduction in fibre volume may indicate that the rate of keratin production becomes sufficient to fill all cortical cells synthesised by the English Leicester follicle. In Drysdale fibres the greater area of medulla \textit{in vivo} results in some unfilled cells remaining even when fibre volume is reduced during \textit{in vitro} growth.

A second hypothesis for the observed differences in medulla production \textit{in vitro} and \textit{in vivo} involves the availability of nutrients. Irrespective of changes in fibre volume, it may be that the nutrient medium in which follicles are maintained \textit{in vitro} provides a level of sulphur amino acids in excess of concentrations available to the follicle \textit{in vivo}. This would allow the synthesis of greater volumes of keratin proteins and therefore a reduction in medulla.

A combination of these two hypotheses probably describes the \textit{in vitro} production of fibres. Nevertheless, the observation that culturing wool follicles affects the degree of fibre medullation may provide an \textit{in vitro} model for the examination of factors responsible for the initiation and maintenance of a medulla \textit{in vivo}.

4.6 \textbf{Discussion and Conclusions}

Fibre growth by wool follicles isolated and maintained in tissue culture is approximately one third of the \textit{in vivo} fibre growth rate. Data in this study suggest that the internal and external structure of the wool fibres are maintained in the cultured follicles. Indeed, fibres produced in the cultured follicles appear identical to \textit{in vivo} fibres as judged by their appearance under low magnification and scanning electron microscopy. This was also verified by comparison of the distance between cuticle ridges of fibres. BrdU uptake verified that typical internal follicle
morphology was maintained in cultured follicles. For example, cell division occurred in the mitotic zone of the follicle bulb surrounding the dermal papilla and in the outer root sheath of the cultured follicle which is identical to the site of BrdU uptake described for follicles in vivo (Adelson et al. 1990; Ibraheem et al. 1993a). Polymerase chain reaction amplification of keratin gene DNA showed that structurally intact DNA is present within follicle cells post-culture.

The reduced growth rate by isolated follicles concurs with most reports on fibre growth in cultured follicles. For example, Ibraheem et al. (1993a) reported an in vitro fibre growth rate for Angora goat follicles of approximately two thirds the in vivo rate. Bond et al. (1992) noted that fibre growth from follicles cultured from Merino sheep was about half the in vivo rate. Hynd et al. (1992) also observed that the rate of fibre growth in vitro for both wool and rat hair follicles failed to approach normal values. Similarly, Waldon et al. (1993) observed that rat whisker follicles were produced in vitro at between 18-50% of the in vivo fibre growth rate. In contrast, Philpott et al. (1989) reported that the rate of human hair growth in vitro approximated the in vivo rate.

This reduction in fibre growth may be due to the lack of factors such as a cytokine (Ebling and Hale 1983; Jahoda et al. 1984), or to loss of structural components required for cell-cell recognition and growth (Jahoda et al. 1984; Odland 1983). The reduced in vitro growth rate suggests there is potential for increased wool production if the stimulatory factors can be identified. Had the growth rate been equivalent to in vivo rates of wool production, it may be that the rate of wool growth in tissue culture was maximal, and that an increase due to experimental follicle stimulation would be unlikely. This is clearly not the case in the in vitro model of wool growth, which therefore has potential for the detection of both stimulatory and inhibitory treatment effects.

The observed correlation between fibre growth and follicle bulb diameter supports other evidence that wool production varies with follicle size (Schinckel 1961; Kelly et al. 1993). This seems reasonable since the larger follicle bulbs had a greater trichocyte population and therefore a greater capacity to produce keratin, and consequently greater fibre production. This study has also shown that follicles which produce fibres at a greater rate (~550μm per day, in vitro) have a stronger correlation with follicle bulb size than follicles which produce fibre at a slower rate. This may represent differential control of primary and secondary follicle populations.

Given that the in vitro growth rate of fibres is correlated with follicle bulb diameter, the use of bulb diameter as a covariate for analysis of variance statistics would be desirable in future work. In this thesis, the randomised design of experiments enabled the assumption that similar sized follicles were isolated for control and treatment groups. Evaluation of follicle bulb size was therefore unnecessary.

It was also shown that medullation was lost when follicles from some sheep breeds were maintained in vitro. This may result from a reduction in fibre volume in concert with an increase in nutrient availability. Wool growth in the absence of any nutrients was also evaluated, in this
study. It was verified that fibre production resulted from a response to the nutrient environment provided by the culture medium, rather than a reflection of processes already underway in the follicle prior to isolation.

In summary, the results show that cultured wool follicles produce a fibre which is structurally similar to the *in vivo* fibre, grows at a measurable rate, and is dependent on the nutrient medium. The *in vitro* wool follicle model developed in this study, therefore provides a unique opportunity to evaluate factors which affect wool growth in an environment which is isolated from the systemic influences of the sheep.
Those who have never entered upon scientific pursuits know not a tithe of the poetry with which they are surrounded.

Herbert Spencer (1820-1903)
English philosopher
5 OPTIMISATION OF THE CULTURE CONDITIONS FOR ISOLATED WOOL FOLLICLES

5.1 Introduction

5.2 An Evaluation of Culture Temperature
   5.2.1 Experimental procedure
   5.2.2 Results and discussion

5.3 Composition of the Basal Tissue Culture Medium
   5.3.1 Experimental procedure
   5.3.2 Results and discussion

5.4 Supplementation of the Basal Culture Medium
   5.4.1 Experimental procedure
   5.4.2 Results
   5.4.3 Discussion

5.5 Supplementation with Foetal Calf Serum
   5.5.1 Experimental procedure
   5.5.2 Results and discussion

5.6 Discussion and Conclusions
5 **OPTIMISATION OF THE CULTURE CONDITIONS FOR ISOLATED WOOL FOLLICLES**

5.1 **INTRODUCTION**

Experimental conditions for the successful growth of human hair in organoid culture were first defined by Philpott *et al.* (1990). Their technique is simple, inexpensive and provides for the maintenance of human hair follicles which retain their typical morphology and physiology. While the conditions described are suitable for the maintenance of human hair follicles *in vitro*, they have not been shown to be optimal for the maintenance of fibre follicles from other species; in particular, they are not necessarily optimal for the growth of wool *in vitro*.

There are numerous examples in the literature detailing different conditions for the maintenance of fibre follicles *in vitro* (for example Davidson and Hardy 1952; Frater and Whitmore 1973; Frater and Hewish 1981; Messenger 1984; Rogers *et al.* 1987). Each presents different growth conditions and reports a varying degree of success with regard to fibre production. Development of an appropriate model of wool fibre growth requires that the initial conditions of Philpott *et al.* (1990), used to date in this study, are optimal for wool production *in vitro*. In this chapter, an evaluation of the temperature at which follicles were maintained, nutrient medium composition and tissue culture supplements was undertaken to optimise conditions for the maintenance of wool follicles *in vitro*.

5.2 **AN EVALUATION OF CULTURE TEMPERATURE**

*In vitro* incubation temperature appears to be a critical factor in the optimisation of epidermal cell growth. Inter-species variability has been reported with mouse epidermal cells demonstrating optimal growth at 31°C in preference to 37°C. However human epidermal cells exhibited a four times greater growth rate at the higher temperature (Fischer *et al.* 1980). This preference for a reduced culture temperature by murine cells was also noted by Imai *et al.* (1991) who reported that murine hair follicles *in vitro* did not maintain their morphology at 37°C and were more viable at 31°C. In contrast, Frater and Whitmore (1973) and Rogers *et al.* (1987) reported the successful maintenance of murine follicles at 37°C. Kondo *et al.* (1990) maintained human hair follicles in culture at 36°C whereas Philpott *et al.* (1989) achieved success with these fibre follicles at 37°C.

Previous data presented in this study showed that wool growth occurs *in vitro* at 37°C. However wool growth at 31°C, a temperature which approximates the observed skin surface temperature, was evaluated in this section to elucidate the optimal temperature for wool production *in vitro*. 
5.2.1 Experimental Procedure

Wool follicles were isolated from the midside of one Romney sheep and maintained in Control Medium A at either 37°C or 31°C. The rate of fibre growth and follicle viability were determined for follicles at both incubation temperatures.

5.2.2 Results and Discussion

The survival of follicles in vitro was significantly reduced ($P < 0.01$) when they were maintained at 31°C compared to 37°C. At the lower temperature only 24% of follicles remained viable and produced a wool fibre. This data is presented in Table 5-1.

<table>
<thead>
<tr>
<th>Culture temperature</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± S.E.M.) ($\mu$m/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>31°C</td>
<td>41</td>
<td>10**</td>
<td>82 ± 25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>37°C</td>
<td>43</td>
<td>40</td>
<td>338 ± 26</td>
<td></td>
</tr>
</tbody>
</table>

Table 5-1 Fibre growth by follicles maintained in Control Medium A at 31°C or 37°C. The difference in the proportion of viable follicles was significant at $P < 0.01$ (**).

Wool growth in vitro at 37°C exceeded that at 31°C. Of those follicles which remained viable, fibre production at 31°C was only one quarter the rate observed by follicles maintained at 37°C. This gives a clear indication that 37°C is a preferential temperature for the maintenance of wool follicles in vitro.

In vivo, wool growth rates have been shown to respond to changes in skin temperature (Coop 1953; Bennett et al. 1962; Hopkins and Richards 1979). This effect may be mediated through growth factors (Wallace 1979), or by a change in blood flow to the skin (Setchell and Waites 1965). The results presented here show that a reduction in follicle temperature directly affects wool production. This provides support for a localised effect of reduced skin temperature on wool follicle activity and fibre synthesis, rather than mediation by blood flow.

5.3 Composition of the Basal Tissue Culture Medium

A complete tissue culture medium consists of a nutrient mix that satisfies the basic metabolic needs of the cell, plus supplements that meet specific growth requirements. A basal nutrient medium contains inorganic salts, essential and non-essential amino acids, vitamins, sugars, lipids, purines, pyrimidines, and polyamines and is generally sufficient to support cell survival and viability (Norman et al. 1989). The composition of the culture medium significantly affects the growth of epidermal tissue in vitro (Thompson et al. 1985). It therefore seems likely that the choice of culture medium will influence the success of growing wool in organoid culture. Philpott et al. (1989, 1990) describe the growth of human hair in William’s Medium E. Dulbecco’s Modified Eagle’s Medium has also been used to culture human hair follicles (Kondo et al. 1990) and Medium 199 for the maintenance of follicle dermal papilla (Messenger 1984). To
5.3.1 **EXPERIMENTAL PROCEDURE**

Isolated wool follicles were maintained in 500μl of William's Medium E, Dulbecco's Modified Eagle's Medium (DMEM), DMEM/Ham's Nutrient Mixture F-12 (DMEM/F12) or Medium 199. Each medium was supplemented with insulin, transferrin, sodium selenite, hydrocortisone, Fungizone®, penicillin/streptomycin and trace elements as detailed in Chapter 3 of this study. This experiment was undertaken on two separate days and the rate of fibre growth, and the proportion of follicles which remained viable, were compared for the different nutrient media on the two occasions.

5.3.2 **RESULTS AND DISCUSSION**

Wool follicle viability was affected by the nutrient medium in which the follicles were maintained. At least three quarters of follicles maintained in William's Medium E, DMEM/F12 and Medium 199 remained viable whereas follicle viability in DMEM was reduced to 38% on Day one and 56% on Day two. These differences in the proportion of viable follicles in DMEM were significant at $P < 0.01$ and $P < 0.1$ for Day one and two respectively, using $\chi^2$ analysis. The data collected in this study are presented in Table 5-2.

<table>
<thead>
<tr>
<th>Day 1</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± S.E.M.) (μm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>William's Medium E</td>
<td>7</td>
<td>7</td>
<td>$188 \pm 40$</td>
<td>0.95</td>
</tr>
<tr>
<td>DMEM</td>
<td>8</td>
<td>3**</td>
<td>$143 \pm 36$</td>
<td>0.36</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>10</td>
<td>9</td>
<td>$220 \pm 28$</td>
<td>0.24</td>
</tr>
<tr>
<td>Medium 199</td>
<td>8</td>
<td>8</td>
<td>$176 \pm 29$</td>
<td>0.60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 2</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± S.E.M.) (μm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>William's Medium E</td>
<td>23</td>
<td>18</td>
<td>$238 \pm 26$</td>
<td>0.27</td>
</tr>
<tr>
<td>DMEM</td>
<td>23</td>
<td>13*</td>
<td>$171 \pm 19$</td>
<td>0.06</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>24</td>
<td>18</td>
<td>$192 \pm 26$</td>
<td>0.38</td>
</tr>
<tr>
<td>Medium 199</td>
<td>25</td>
<td>19</td>
<td>$234 \pm 25$</td>
<td>0.32</td>
</tr>
</tbody>
</table>

*Table 5-2 Wool growth by follicles maintained in different nutrient media. Significant between treatment differences in the proportion of viable follicles are indicated ( * $P < 0.1$, ** $P < 0.01$ ).

In addition to the observed decrease in follicle viability, there was also a reduction in the rate of fibre growth by follicles maintained in DMEM. There was no difference in the rate of wool production in vitro by follicles maintained in supplemented DMEM/F12, Medium 199 and William's Medium E. However the maintenance of follicles in DMEM suppressed the rate of fibre growth by approximately 25%. Values given in this study, showing the probability of statistical
differences between treatments (P values), relate to the rate of fibre growth by all the other follicles isolated on that day.

DMEM is deficient in a number of components, compared with the other nutrient media examined here. Of the amino acids, alanine, asparagine, aspartic acid, cysteine, glutamic acid and proline are absent from DMEM. The most critical of these for wool growth is the sulphur amino acid, cysteine which has been shown to be essential for wool growth in vivo (Ryder 1958; Reis and Tunks 1978; Reis 1979; Hemsley and Reis 1984). In support of these in vivo findings, Nancarrow and Hynd (1994) demonstrated that lysine, methionine, cyst(e)ine, leucine and glutamine are essential for normal fibre production by wool follicles in culture.

The deficiency of the amino acid cysteine, in DMEM, may be responsible for the observed reduction in viability and wool growth in vitro, however numerous other components are also deficient in this media. For example ascorbic acid, biotin, vitamin B-12, vitamin E and the vitamin A derivative, retinol acetate are not present in DMEM. The vitamin composition of each of the nutrient media is shown in Table 5-3. Biotin is absent only in DMEM. Ryder (1958) has shown that biotin is essential for wool growth in vivo, therefore the potential of biotin to perform a regulatory role in wool production deserves future evaluation.

<table>
<thead>
<tr>
<th>VITAMINS</th>
<th>William's Medium E (mg/ml)</th>
<th>Medium 199 (mg/ml)</th>
<th>DMEM (mg/ml)</th>
<th>DMEM/F12 (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>0.002</td>
<td>0.0000566</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-biotin</td>
<td>0.0005</td>
<td>0.00001</td>
<td></td>
<td>0.0000035</td>
</tr>
<tr>
<td>Calciferol</td>
<td>0.0001</td>
<td></td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.0015</td>
<td>0.0005</td>
<td>0.004</td>
<td>0.00898</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.001</td>
<td>0.00001</td>
<td>0.004</td>
<td>0.00265</td>
</tr>
<tr>
<td>myo-inositol</td>
<td>0.002</td>
<td>0.00005</td>
<td>0.0072</td>
<td>0.0126</td>
</tr>
<tr>
<td>Menadione</td>
<td>0.00001</td>
<td></td>
<td>0.00016</td>
<td></td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>0.00025</td>
<td>0.004</td>
<td>0.00202</td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td></td>
<td>0.000025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Amino benzoic acid</td>
<td></td>
<td>0.00005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-pantothenic acid</td>
<td>0.001</td>
<td>0.00001</td>
<td>0.004</td>
<td>0.00224</td>
</tr>
<tr>
<td>Pyridoxal.HCl</td>
<td>0.001</td>
<td>0.000025</td>
<td>0.004</td>
<td>0.002</td>
</tr>
<tr>
<td>Pyridoxine.HCl</td>
<td></td>
<td>0.000025</td>
<td></td>
<td>0.00031</td>
</tr>
<tr>
<td>Retinol acetate</td>
<td>0.0001</td>
<td>0.00014</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.0001</td>
<td>0.00001</td>
<td>0.004</td>
<td>0.00219</td>
</tr>
<tr>
<td>Thiamine.HCl</td>
<td>0.001</td>
<td>0.00001</td>
<td>0.004</td>
<td>0.00217</td>
</tr>
<tr>
<td>DL-a-tocopherol phos</td>
<td>0.00001</td>
<td>0.00001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B-12</td>
<td>0.0002</td>
<td></td>
<td></td>
<td>0.00068</td>
</tr>
</tbody>
</table>

Table 5-3 The vitamin composition of the four nutrient media examined in this study for the in vitro growth of wool fibres. Biotin (highlighted) is absent only from DMEM. (Sigma Cell Culture Catalogue).

It may be that any one, or a combination of mechanisms is responsible for the observed reduction in follicle viability and fibre growth in follicles cultured in DMEM. The wool follicle culture model provides an opportunity for researchers to identify factors involved in the control of wool growth by supplementing the deficient medium with potential growth regulators.
For the optimisation of fibre growth, William's Medium E, DMEM/F12 and Medium 199 appear equally suitable as the culture medium. In this study, William's Medium E was selected on the grounds of compatibility with the medium used by other workers (Philpott et al. 1989; Ibraheem et al. 1993a,b; Bond et al. 1994a).

### 5.4 Supplementation of the Basal Culture Medium

Supplementation of the basal nutrient medium occurs in almost every in vitro culture system (Breidahl et al. 1989). It is the specific supplements added to the medium that often determines cell behaviour. Insulin, hydrocortisone, sodium selenite, transferrin, trace elements and antibiotics are typical supplements and are included in the method reported by Philpott et al. (1989) for the human hair follicles. Each of these supplements is included in the Control Medium A used in this study. However the requirement of cultured wool follicles for these specific supplements has not been determined. Possible functions of the supplements for in vivo and in vitro wool production are discussed below.

Insulin is an endocrine growth factor synthesised primarily in response to high blood glucose. Neither its synthesis or utilisation have been identified in wool follicle tissues. Bray et al. (1990) suggested an energy partitioning role for insulin in wool fibre production. This has yet to be demonstrated. Furthermore, a number of in vivo studies have shown that wool growth can be independent of changes in systemic insulin concentrations (Johnsson et al. 1985; Wynn et al. 1988; Bray et al. 1990). In tissue culture, insulin has been reported to induce many metabolic changes including stimulation of hexose, carbohydrate, amino acid and ion transport, together with glycogen and protein synthesis. The responses have been variable depending on cell type (Norman et al. 1989). Conflicting findings have been reported (Fischer et al. 1980; Wilkinson et al. 1987; Breidahl et al. 1989) and the effects of insulin in culture remain undefined.

Nevertheless, most tissue culture systems continue to supplement nutrient medium with insulin. Weterings et al. (1982), Boyce and Ham (1983), Maurer (1986) and Philpott et al. (1989) state that insulin is essential for the growth of nearly all cells in culture. In this study, the effect of insulin on in vitro wool growth was investigated.

Hydrocortisone is also usually incorporated in tissue culture systems with variable effects on growth stimulation being reported (Hawley-Nelson et al. 1980; Macalg and Nemore 1981). An optimal concentration for hydrocortisone has not been determined, consequently a range of values (10ng/ml - 0.5μg/ml) have been recommended (Thompson et al. 1985, Philpott et al. 1989, Kondo et al. 1990). Hynd et al. (1994) identified that 0-1000ng/ml cortisol has no effect on wool fibre growth in vitro. In contrast, Westgate et al. (1993) found hydrocortisone to be necessary for the maintenance of viable human hair follicles in vitro. The effect of hydrocortisone on wool follicle viability and fibre growth was investigated in this chapter.

Selenium is typically included in the culture medium for a number of epithelial cell types and is thought to be essential for the activation of glutathione reductase, a key enzyme involved in oxygen metabolism, and thus energy production (Maurer 1986). Selenium is also a co-factor for
glutathione peroxidase which protects cells against oxidation damage (Norman et al. 1989). The composition of the media described by Philpott et al. (1989) incorporates sodium selenite, however later work indicated that selenium was not essential for human hair growth under in vitro conditions (Westgate et al. 1993).

Transferrin is another factor which is usually included in culture media, particularly serum free media (Norman et al. 1989). Transferrin binds to a specific receptor on the cell surface and is involved in cellular iron transport (Lehninger 1975). Transferrin has been described as essential for most cells in tissue culture (Maurer 1986; Norman et al. 1989). However in vitro human hair growth, in the absence of transferrin supplementation, has recently been reported (Westgate et al. 1993). The growth of wool in tissue culture, without transferrin, was evaluated in this chapter.

The requirement of isolated follicles maintained in vitro, for trace elements, has not been investigated. Control Medium A incorporates numerous trace elements through the addition of the trace element mix as described by Philpott et al. (1989). The specific purpose for each of these elements has not been defined. This study examines the effects of trace element supplements on wool follicle growth.

Most epidermal and follicle organ culture systems are supplemented with penicillin and streptomycin (for example Messenger 1984; Philpott et al. 1989; Kondo et al. 1990). It has been demonstrated, however, that epidermal cells are extremely sensitive to the toxic effect of antibiotics, particularly gentamycin and Fungizone® (Noyes et al. 1979). This suggests that the removal of Fungizone® and penicillin/streptomycin from Control Medium A may affect the growth of wool fibres in vitro. Removal may also leave the system vulnerable to microbial contamination.

5.4.1 EXPERIMENTAL PROCEDURE

Previous work presented in this study has evaluated wool fibre growth and follicle viability in nutrient medium supplemented with insulin, hydrocortisone, sodium selenite, transferrin, trace elements and antibiotics, following the method detailed by Philpott et al. (1989). An attempt was made here to determine which of these supplements are actually required for the maintenance of viable wool follicles in vitro. This was achieved through a series of experiments in which all, or some, of the supplements have been removed. In each experiment, the rate of wool growth and the viability of follicles was assessed and compared with that occurring with Control Medium A.

The following series of experiments was undertaken, with treatment effects sometimes examined on more than one occasion.

**Experiment one** - William’s Medium E with no additional supplements

**Experiment two** - William’s Medium E supplemented with only insulin (10μg/ml) and hydrocortisone (10ng/ml).
Experiment three - William's Medium E supplemented with insulin (10μg/ml), hydrocortisone (10ng/ml) and sodium selenite (10ng/ml).

Experiment four - William's Medium E supplemented with all the factors except insulin

Experiment five - William's Medium E supplemented with all the factors except trace element mix.

Experiment six - William's Medium E supplemented with all factors except the antimycotic Fungizone®, and the antibiotics, penicillin and streptomycin.

5.4.2 RESULTS

The effect of the various supplement mixtures, on wool growth, are shown in Table 5-4.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± S.E.M.) (μm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment one Day one Control Medium A</td>
<td>8</td>
<td>7</td>
<td>85 ± 23</td>
<td>0.005</td>
</tr>
<tr>
<td>Unsupplemented medium</td>
<td>12</td>
<td>12</td>
<td>207 ± 29</td>
<td></td>
</tr>
<tr>
<td>Day two Control Medium A</td>
<td>23</td>
<td>17</td>
<td>179 ± 19</td>
<td>0.97</td>
</tr>
<tr>
<td>Unsupplemented medium</td>
<td>21</td>
<td>18</td>
<td>148 ± 14</td>
<td>0.2</td>
</tr>
<tr>
<td>Experiment two Control Medium A</td>
<td>44</td>
<td>39</td>
<td>273 ± 23</td>
<td>0.12</td>
</tr>
<tr>
<td>insulin + hydrocortisone</td>
<td>46</td>
<td>44</td>
<td>227 ± 19</td>
<td></td>
</tr>
<tr>
<td>Experiment three Control Medium A</td>
<td>24</td>
<td>24</td>
<td>362 ± 35</td>
<td>0.97</td>
</tr>
<tr>
<td>insulin + hydrocortisone + sodium selenite</td>
<td>23</td>
<td>20</td>
<td>359 ± 40</td>
<td></td>
</tr>
<tr>
<td>Experiment four Control Medium A</td>
<td>24</td>
<td>22</td>
<td>183 ± 22</td>
<td>0.90</td>
</tr>
<tr>
<td>no insulin</td>
<td>22</td>
<td>15*</td>
<td>187 ± 23</td>
<td></td>
</tr>
<tr>
<td>Experiment five Control Medium A</td>
<td>17</td>
<td>14</td>
<td>144 ± 29</td>
<td>0.35</td>
</tr>
<tr>
<td>no trace element mix</td>
<td>16</td>
<td>14</td>
<td>183 ± 26</td>
<td></td>
</tr>
<tr>
<td>Experiment six Control Medium A</td>
<td>46</td>
<td>37</td>
<td>136 ± 17</td>
<td>0.016</td>
</tr>
<tr>
<td>no antibiotic/antimycotic</td>
<td>42</td>
<td>38</td>
<td>206 ± 22</td>
<td></td>
</tr>
</tbody>
</table>

Table 5-4 Fibre growth by cultured wool follicles maintained in William's Medium E with various combinations of supplements. Differences in the proportion of viable follicles were determined using χ² analysis; *P < 0.05.

In Experiment one, the growth of follicles maintained in unsupplemented William's Medium E was evaluated on two occasions; Day one and Day two. Follicle viability was unaffected by the
exclusion of all supplements from the control medium, with between 75 -100% of follicles remaining viable on both days. With regard to the rate of wool growth, on Day one there was a significantly greater rate of fibre growth by follicles maintained in unsupplemented William's Medium E than those maintained in Control Medium A (P=0.005). In contrast, on Day two, follicles in Control Medium A produced fibre at a slightly greater rate than follicles in William's Medium E (P=0.2). These results support the longevity data for this experiment in that follicle longevity was improved by the removal of supplements on Day one (6.5±1.0 days for unsupplemented, 3.1±0.6 days for control follicles (mean±S.E.M.), P<0.01). Longevity data was not calculated for Day two.

Because of the conflicting results, it is difficult to clearly identify the effect of maintaining follicles in William's Medium E without supplements. It is apparent that wool production occurs when follicles are maintained in William's Medium E. Any additional supplements may, in fact, be detrimental to the rate of fibre growth. It may be that some supplements are essential for fibre growth, but that the effect of supplementation is overshadowed by other detrimental factors. The evaluation of fibre growth in the absence of some supplements is studied in experiments two to six.

At least 85% of isolated wool follicles remained viable when maintained in either Control Medium A or William's Medium E supplemented with insulin and hydrocortisone. As shown in Table 5-4 (experiment two), the growth rate of fibres by follicles maintained in the treatment medium was less than that by follicles maintained in Control Medium A. This difference was not significant (P=0.12). The data suggest that there may be a slight detrimental effect of including only insulin and hydrocortisone in the basal medium.

When sodium selenite was included with insulin and hydrocortisone (experiment three, Table 5-4), there was no difference in the rate of fibre growth between treatment and control follicles (P=0.97). There was also no difference in the proportion of follicles which remained viable. The results indicate that the supplementation of William's Medium E with insulin, hydrocortisone and sodium selenite provides adequate conditions for wool fibre growth in vitro.

Supplementation of William's Medium E with all the components except insulin (experiment four, Table 5-4) had no effect on the rate of fibre growth. Follicle viability was, however, inhibited (P<0.05). This study indicates that while supplementation of the nutrient medium with insulin is not essential for the production of a wool fibre, it may be involved in maintaining follicles in anagen.

In experiment five (Table 5-4), the inclusion of the trace element mix in Control Medium A had a slight, but non-significant, tendency to inhibit fibre growth (P=0.35). No effect of trace element supplementation on follicle viability was observed. There appears to be no advantage in supplementing William's Medium E with the trace element mix used in this study.

Follicles maintained in the absence of Fungizone®, penicillin and streptomycin produced wool fibre in vitro at a significantly greater rate than follicles maintained in basal medium supplemented with these factors (P=0.016). These results are shown in Table 5-4 (experiment six). There
was also a tendency towards increased follicle viability for the group of follicles maintained in the absence of antibiotics and antmycotics ($P = 0.18$).

The exclusion of antibiotics and antmycotics from the basal medium had little effect on the rate of microbial contamination in this tissue culture system. Typically less than 10% of follicles became contaminated in the absence of these factors compared with no apparent contamination when antibiotics and antmycotics were included. Ten percent was considered to be an acceptable degree of contamination given the significant depressive effect on the rate of fibre growth when antibiotics and antmycotics were included.

An analysis of follicle viability and fibre growth in the presence or absence of microbial contamination showed that there was a significant reduction in the rate of fibre growth when bacteria or fungus were present ($P < 0.001$). These data are presented in Table 5-5. Presence of microbial contamination was determined using nutrient agar. The viability of follicles in vitro was unaffected by the presence of any microbial contaminant.

<table>
<thead>
<tr>
<th>Bacteria / fungus</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± S.E.M.) (μm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>absent</td>
<td>57</td>
<td>55</td>
<td>260±18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>present</td>
<td>50</td>
<td>44</td>
<td>116±10</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5-5** Effect of microbial contamination of the culture medium, on wool production.

### 5.4.3 DISCUSSION

It has not been possible to conclusively evaluate the requirement of follicles in culture for the supplements insulin, hydrocortisone, sodium selenite and transferrin. In separate experiments conflicting results were obtained. William's Medium E devoid of all supplements proved highly beneficial in one experiment (250% growth rate of controls and twice the longevity), however in a second experiment only 80% of the control growth rate was observed. No consistent results were obtained with the removal of specific potential growth factors. However follicles maintained in the absence of trace elements grew fibre at 130% the rate of control follicles. Therefore there seems to be no advantage in using trace elements in addition to those already present in William's Medium E. Removal of antibiotics and antmycotics from the nutrient medium also resulted in a significant growth rate improvement (150% of controls). From these results it seems reasonable to maintain follicles in William's Medium E supplemented with insulin, hydrocortisone, sodium selenite and transferrin.

The occurrence of bacteria or fungus during maintenance of follicles in vitro results in a severe reduction in the wool growth rate to 45% of controls. The exclusion from statistical analysis, of follicles with microbial contamination, is therefore desirable and was used for all experiments in this thesis.
5.5 **Supplementation with Foetal Calf Serum**

Mammalian blood and its components, plasma and serum, contain many different growth factors. Serum has been widely used to promote *in vitro* cell growth, although serum is an undefined and complex mixture of hormones, growth factors, attachment factors, transport factors and essential nutrients (Norman *et al.* 1989). In cell culture it is desirable to develop a serum free system in order to study the effects of supplementary growth factors without the confounding effects of endogenous serum growth factors.

In many dispersed cell culture systems a dependence on serum supplementation has been demonstrated (Maciag and Nemore 1981; Moore 1988). However a major role of serum in these systems is the provision of unidentified attachment and spreading factors (Maurer 1986) that are not required for the maintenance of whole organs in culture. During the organ culture of various fibre follicles it has been shown that serum may enhance (Kondo *et al.* 1990), have no effect (Boyce and Ham 1983) or inhibit (Philpott *et al.* 1990) fibre growth. As fibre production does occur in the absence of serum, it is possible that the autocrine or paracrine forms of growth factors exist in the dermal papilla or other trichocytes (Kondo *et al.* 1990). In regard to the inhibitory effects of serum, one hypothesis involves polyamine oxidase. As noted in Chapter 2, this enzyme is found in ruminant serum and results in the formation of cytotoxic polyaminoaldehydes (Maurer 1986) and hydrogen peroxide (Williams-Ashman and Canellakis 1979).

Human hair has been successfully grown in serum free William's Medium E. The system also demonstrated the inhibitory effects of supplementary foetal calf serum (Philpott *et al.* 1990). In contrast, fibre growth in DMEM was enhanced by supplementation with foetal calf serum (Kondo *et al.* 1990). A number of differences exist in the composition of these two media. For example, William's Medium E has additional amino acids and vitamins compared to DMEM. Specific reasons for the effects of supplementation of DMEM with serum have not been reported. Control Medium A does not incorporate foetal calf serum, however the hypothesis that it's inclusion will affect the rate of wool production *in vitro* is of interest and requires investigation.

5.5.1 **Experimental Procedure**

Wool follicles were isolated from the midside of one Romney sheep and maintained in Control Medium A. A second group of isolated follicles were maintained in Control Medium A supplemented with 1% foetal calf serum (Cytosystems, Aust.). The rate of wool growth and viability of isolated follicles was compared, for the two follicle groups.

5.5.2 **Results and Discussion**

Supplementation of Control Medium A with 1% foetal calf serum did not affect the rate of fibre growth. Results from this study are presented in Table 5-6. The longevity of wool follicles was also unaffected by serum supplementation (4.0±0.2 days for serum supplemented and 4.4±0.2
days for control follicles, mean ± S.E.M.). There was no difference in the proportion of viable follicles between the two groups.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± S.E.M.) (µm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Medium A</td>
<td>33</td>
<td>30</td>
<td>157 ± 10</td>
<td></td>
</tr>
<tr>
<td>1% foetal calf serum</td>
<td>32</td>
<td>28</td>
<td>182 ± 18</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Table 5-6 Wool production of follicles maintained in Control Medium A in the presence or absence of foetal calf serum.

Supplementation of the control medium with 1% foetal calf serum did not alter the rate of in vitro wool growth, in the study presented here. This is inconsistent with the findings of Westgate et al. (1993) who demonstrated that 1 and 10% foetal calf serum inhibited hair growth in vitro. Foetal calf serum may be expected to inhibit fibre production due to growth factors present in serum, such as transforming growth factor-β, which has been shown to be a potent inhibitor of hair fibre growth (Philpott et al. 1990). Alternately cellular biochemical pathways may be disrupted due to the presence of enzymes such as polyamine oxidase which is present in foetal calf serum and actively oxidises the polyamines spermine and spermidine, essential for wool growth, into cytotoxic polyaminoaldehydes (Williams-Ashman and Canellakis 1979).

A major role for foetal calf serum in tissue culture systems is the provision of attachment and spreading factors, which are not required in an organoid culture system. Given these observations, and the desirability of maintaining follicles in serum free medium to enable evaluation of supplementary growth factor effects, distinct from those present in serum, there is no advantage in supplementing the basal medium with foetal calf serum.

It would be interesting to examine effects of sheep serum on wool growth by isolated follicles. Hibberts and Randall (1992) note that dermal papilla cells isolated from human hair follicles were healthier and grew at a faster rate when supplemented with human rather than foetal calf serum. An evaluation of the effects of ovine serum on the fibre growth rate of isolated wool follicles is undertaken in Chapter 7 of this study.

5.6 DISCUSSION AND CONCLUSIONS

The data presented in this study show that the conditions recommended by Philpott et al. (1989) for the growth of human hair in vitro, are not optimal for the maintenance of wool follicles in tissue culture. Later work by these authors specified a defined medium supplemented with only antibiotics, insulin and hydrocortisone for the growth of human hair in vitro (Westgate et al. 1993). The data presented here show that wool follicles can be successfully maintained in William's Medium E with no additional supplements. Nonetheless, it seems reasonable to include insulin, hydrocortisone, sodium selenite and transferrin due to possible detrimental effects if these factors are removed. Indeed, the absence of sodium selenite and insulin leads to a reduction in the rate of wool growth or follicle viability respectively.
Supplementary trace elements and Fungizone®, penicillin and streptomycin are disadvantageous to wool growth *in vitro*.

Wool growth *in vitro* was successful in each of William's Medium E, DMEM/F12 and Medium 199. In this study, wool follicles will be maintained in William's Medium E to enable comparisons with other literature results on the effects of mitogens on fibre growth. The rate of wool growth and the viability of wool follicles *in vitro* at 37°C exceeded that of follicles maintained at 31°C. Thus, all future experiments will be undertaken at 37°C.

One factor which was not evaluated in this study was the inclusion of fatty acids. Wool follicles lie in the skin with the bulb next to the dermal fat layer. It is, therefore, possible that fat soluble factors may influence follicle growth. Fatty acids are insoluble in the culture medium and require a protein carrier. The inclusion of fatty acids could be evaluated in future studies.

In conclusion, optimal conditions for the maintenance of ovine wool follicles *in vitro* are provided by Control Medium B, defined as William's Medium E supplemented with insulin, hydrocortisone, sodium selenite and transferrin. Isolated follicles should be maintained at 37°C. Individual follicles which are contaminated with bacteria or fungus should not be included in the statistical calculation of treatment effects due to the significant depressive influence on the rate of fibre growth observed under these conditions.
Though science can cause problems, it is not by ignorance that we will solve them.

Isaac Asimov
6 AN EVALUATION OF *IN VITRO* WOOL GROWTH FOR SHEEP OF DIFFERENT GENOTYPES

6.1 Introduction

6.2 Experimental Procedure

6.3 Results

6.4 Discussion and Conclusions
6 AN EVALUATION OF IN VITRO WOOL GROWTH FOR SHEEP OF DIFFERENT GENOTYPES

6.1 INTRODUCTION

Sheep genotype is a major factor influencing wool growth in vivo. Clean wool production, fibre diameter and the seasonal variation of wool growth vary significantly with sheep breed (Bigham et al. 1978). Ross (1990) has reviewed breed differences, and Table 6-1 details typical ranges for fibre diameter and wool growth rate for some common sheep breeds in New Zealand. Annual staple length gives an indication of the amount of wool produced each year. The average fibre growth rate is calculated from these data to determine daily growth rates. While average daily growth is likely to be a reasonable estimate for breeds that show little seasonal variation (for example Merino), the figure will not accurately reflect daily fluctuations for breeds that have seasonal variations in wool growth (for example English Leicester).

<table>
<thead>
<tr>
<th>Sheep breed</th>
<th>Fibre diameter (µm)</th>
<th>Annual staple length (mm)</th>
<th>Average fibre growth rate (µm/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finewool - Merino</td>
<td>18-25</td>
<td>60-100</td>
<td>220</td>
</tr>
<tr>
<td>- Crossbred</td>
<td>25-31</td>
<td>75-125</td>
<td>270</td>
</tr>
<tr>
<td>Downwool - Poll Dorset</td>
<td>27-32</td>
<td>75-100</td>
<td>250</td>
</tr>
<tr>
<td>- Suffolk</td>
<td>28-35</td>
<td>75-100</td>
<td>250</td>
</tr>
<tr>
<td>Longwool - Romney</td>
<td>31-41</td>
<td>125-175</td>
<td>410</td>
</tr>
<tr>
<td>- English Leicester</td>
<td>36-42</td>
<td>150-200</td>
<td>480</td>
</tr>
<tr>
<td>Carpetwool - Drysdale</td>
<td>36-49</td>
<td>200-300</td>
<td>680</td>
</tr>
</tbody>
</table>

Table 6-1 Fibre diameter, annual staple length and estimated fibre growth per day for some domestic sheep breeds, adapted from (Ross 1990).

Genotype may also influence wool growth and follicle survival in vitro. The ease with which follicles can be isolated is likely to depend on the toughness of the dermis, the size, evenness and curvature of follicles and the degree to which the follicles are intertwined. These factors vary considerably between sheep breeds. Thus, it seems reasonable that follicles which are easier to isolate are likely to be less damaged and more likely to remain viable, than follicles which are difficult to isolate.

The work presented here examines two related hypotheses:

1. that follicles from coarse wool breeds are easier to isolate than those from finewool breeds and are therefore more likely to remain viable and produce wool fibre in vitro, and
2. that the wool growth rate in vitro correlates with the rate of wool growth in vivo for the different sheep genotypes.
6.2 Experimental Procedure

Skin strips were collected from the midside of one animal from each of seven breeds of sheep. Individual follicles were isolated by microdissection from the skin strips. Fibre production by follicles maintained in Control Medium B for 72 hours was determined by fixed time analysis on four separate days. Follicles were not isolated from all breeds on each day.

6.3 Results

Table 6-2 shows the in vitro wool production data for the seven sheep breeds on each of the four days.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Day one</th>
<th>Day two</th>
<th>Day three</th>
<th>Day four</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>number of follicles isolated</td>
<td>number of follicles viable</td>
<td>fibre growth rate (mean ± S.E.M.) (μm/day)</td>
<td>P value (growth rate)</td>
</tr>
<tr>
<td>Merino</td>
<td>40</td>
<td>14**</td>
<td>54±7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Romney</td>
<td>40</td>
<td>39</td>
<td>155±15</td>
<td></td>
</tr>
<tr>
<td>Drysdale</td>
<td>13</td>
<td>11</td>
<td>154±22</td>
<td>0.87</td>
</tr>
<tr>
<td>Suffolk</td>
<td>14</td>
<td>12</td>
<td>159±30</td>
<td>0.96</td>
</tr>
<tr>
<td>English Leicester</td>
<td>11</td>
<td>11</td>
<td>169±36</td>
<td>0.70</td>
</tr>
<tr>
<td>Poll Dorset</td>
<td>11</td>
<td>5**</td>
<td>137±27</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Table 6-2 Fibre growth in vitro by follicles isolated from different sheep breeds (mean ± S.E.M.). Data from each day was treated independently; *P<0.05, **P<0.01 by χ² analysis for differences in the proportion of viable follicles. Probability values for wool growth differences are relative to all other values for that day.

On Day one, wool growth from follicles of a finewool Merino was approximately one third the rate of cultured follicles isolated from a Romney (P<0.001). Chi squared analysis also showed a significant reduction in the proportion of follicles that remained viable when isolated from a Merino compared to a Romney sheep (P<0.001). That is, 35% of Merino follicles, compared to 98% of Romney follicles, remained viable over a 72 hour culture period.

On Day two, Drysdale, Suffolk and English Leicester follicles produced fibre at similar rates and at least 85% of follicles remained viable. In contrast, Poll Dorset follicles showed a non-significant reduction in growth rate, with only 45% of the follicles remaining viable.
On Day three, follicles from Drysdale, English Leicester and New Zealand Wiltshire sheep produced fibre at a similar rate with approximately 90% of the follicles remaining viable. Similarly, on Day four there was no significant difference in wool growth from follicles isolated from a Romney, Drysdale and English Leicester sheep, although the latter was measurably lower than the other breeds. On Day four only 70% of the follicles remained viable in all the breeds examined.

There was considerable variation in the rate of growth of fibres from follicles on the different days. Growth rates on Day three were considerably higher than on Day two, with the growth rates of Drysdale and English Leicester fibres on the latter day being 150% of those measured on Day two. The reason for this is unknown. Some possible hypotheses are discussed below.

6.4 DISCUSSION AND CONCLUSIONS

Wool follicles from English Leicester and Drysdale animals were easy to isolate relative to other sheep breeds as the follicles lie parallel and straight, in the dermal tissue. Dissection of finewool Merino and downwool Poll Dorset follicles was considerably more difficult than for other sheep breeds due to the greater degree of follicle curvature, and decreased follicle size, observed in the skin of these animals. Other follicles from the downwool breeds, New Zealand Wiltshire and Suffolk, were comparatively easier to isolate as there was less follicle curvature. Romney follicles displayed some curvature making them less easy to isolate than were English Leicester follicles.

The follicle differences can be seen in the following four photographs (Plates 6-1 to 6-4), which show typical skin sections from each of the wool types stained with Nile blue sulphate, using the protocol of Maddocks and Jackson (1988). In these photographs, wool follicles are stained dark blue. Connective tissue and wool fibres remain unstained. Follicle characteristics are illustrated in Plate 6-1 Crossbread (finewool); Plate 6-2 Poll Dorset (downwool); Plate 6-3 English Leicester (longwool); and Plate 6-4 Drysdale (carpetwool). In each of these plates, the skin surface is shown at the top, with follicle bulbs near the bottom of the photograph. Each skin section is magnified 20X.
Plate 6-1  Histological slide showing follicles in the skin of a Crossbred (finewool) sheep (20X magnification).

Plate 6-2  Histological slide showing follicles in the skin of a Poll Dorset (downwool) sheep (20X magnification).
From these photographs shown in Plates 6-1 to 6-4, it can be seen that there are clear differences between the larger primary follicles, and smaller secondary follicles, in Drysdale skin (Plate 6-4). This size difference between follicles has been noted previously (Carter and Clarke 1957b; Black 1987; Orwin 1988). It has also been observed that variations in primary and secondary follicle size are breed-related. Thus, in animals which had obvious differences in follicle size, it is likely that the larger primary follicles were collected during follicle isolation, due
to their relative ease of isolation. This is likely to be true of the New Zealand Wiltshire sheep, which have very coarse guard hairs, or kemps, of up to 67µm diameter, together with fine secondary down fibres of up to 38µm diameter (Carter and Clarke 1957b), and of Drysdale sheep.

Plates 6-1 to 6-3 show that in many other breeds there is no clear difference in the size of primary and secondary follicles. Thus, a mixture of primary and secondary follicles are likely to have been isolated from Merino, Romney, Poll Dorset, Suffolk and English Leicester sheep due to an inability to differentiate between follicle types on the basis of size.

The selection of large primary follicles in some breeds is likely to have had an effect on follicle fibre growth. In Chapter 5 it was demonstrated that follicles with a larger bulb diameter had a tendency to grow at a greater rate than smaller follicles; a trend which has frequently been observed in vivo (reviewed by Black 1987; Orwin 1988). During follicle isolation, the selection of large primary follicles in some breeds is therefore likely to have a positive influence on the observed in vitro fibre growth rate. Finewool and downwool breeds which do not have these large primary follicles are likely to have a similar rate of wool growth in vitro relative to that observed in vivo.

Carter and Clarke (1957b) and Orwin (1988) observed that skin follicle density is breed dependent, with Merino sheep having a very high follicle density (80 per mm²), in comparison with Romney and Drysdale animals, which have approximately 14 follicles per mm². In the experiments described here it was found that follicles which have a greater density in the skin, are more difficult to isolate; a factor which is likely to reduce follicle viability in vitro.

None of the breeds examined fulfilled their apparent genetic potential for fibre growth according to the data presented in Table 6-1. This concurs with the findings of Chapter 4, where it was shown that English Leicester and Drysdale follicles produced wool in vitro at approximately half the rate observed in vivo. Similar growth rate ratios were found in this section for Suffolk, Romney, English Leicester and Drysdale animals. Figure 6-1 shows the relationship between in vivo and in vitro wool growth for sheep of each of the breeds examined in this chapter. Merino follicles had the lowest fibre growth rate both in vivo and in vitro with Drysdale follicles having the highest growth rate under both conditions.
**Figure 6-1** Relationship between *in vivo* and *in vitro* wool growth by sheep of six different breeds. Wool growth *in vivo* was estimated from the data of Ross (1990) presented in Table 6-1.

This correlation of fibre growth rates probably reflects true phenotypic differences in wool growth, rather than differential damage inflicted during dissection. Hynd *et al.* (1992) also observed that relative phenotypic differences between sheep breeds *in vivo* are reflected *in vitro*. This finding is important as it indicates that genetic differences in wool growth are generated at the follicle level, and are not merely a consequence of differences in nutrient supply or hormonal patterns. This concurs with the suggestion of Black (1987), that while factors regulating wool production are genetically controlled, actual values are influenced by the environment of the wool follicle.

There was a significant reduction in the proportion of follicles which survived in organoid culture from Merino and Poll Dorset animals. As discussed earlier, this may be an effect of isolation damage to the follicles themselves due to their relatively small size and high follicle density. Alternatively, animals from these breeds may be highly sensitivity to stress related growth factors released during the collection of skin strips, prior to follicle isolation. Plasma levels of cortisol are elevated in response to stress and Behrendt *et al.* (1993a) demonstrated that an increased plasma cortisol concentration can affect wool growth *in vivo*. However Hynd *et al.* (1994) found no effect of cortisol on the *in vitro* rate of wool production. This latter study does not rule out the possibility of between breed differences. For instance, Behrendt *et al.* (1993) demonstrated that individual animals within one breed may have differences in their sensitivities to stress related growth factors. Thus, it is possible that between breed differences occur. These differences could be examined by investigating the effects of elevated cortisol concentrations on the *in vivo* and *in vitro* rate of wool growth in different breeds of sheep.

There was a significant difference in the growth rate of fibres on consecutive days. For example, Drysdale and English Leicester follicle growth rates were 50% higher on Day three than Day two. The difference in growth rates are difficult to explain since the isolation and growth conditions
The difference in growth rates are difficult to explain since the isolation and growth conditions for the follicles were identical. Some possible explanations are that variability in sheep handling during skin strip biopsy may have affected the plasma concentration of stress related growth factors; alternately, daily environmental conditions, for example temperature, may have varied which affected the hormonal balance within the follicle. Factors such as age of the nutrient medium and the elapsed time between strip collection and follicle isolation, have been monitored and do not appear to be responsible for these differences in follicle production in vitro.

In summary, it is easier to isolate large, straight follicles such as those occurring in the skin of English Leicester and Drysdale sheep. Furthermore, a high proportion of these follicles remain viable in organoid culture. Follicle fibre growth appears to be correlated with bulb size. Consequently, the rate of fibre growth by these large follicles is relatively high. Excessive curvature, small size and high density make follicle isolation difficult and leads to reduced viability in vitro. Effects of stress related hormones on follicle fibre growth between breeds and between different days of isolation, cannot be discounted. The rate of wool production in vitro is moderately correlated to in vivo wool growth rates. As follicles from coarse wool breeds are easier to isolate than those from fine wool breeds and are more likely to remain viable and produce wool fibre in vitro, coarser wool breeds of sheep are recommended for in vitro studies of wool growth.
CHAPTER SEVEN

AN EVALUATION OF THE EFFECT OF OVINE SERUM ON WOOL GROWTH AND FOLLICLE VIABILITY IN VITRO

The most exciting phrase to hear in science, the one that heralds new discoveries, is not “Eureka: I found it!” but “that’s funny.....”

Isaac Asimov
7 AN EVALUATION OF THE EFFECT OF OVINE SERUM ON WOOL GROWTH AND FOLLICLE VIABILITY IN VITRO

7.1 Introduction

7.2 Effects of Adult Ovine Serum on Wool Growth by Follicles Maintained In Vitro
   7.2.1 Experimental procedure
   7.2.2 Results and discussion

7.3 Fractionation of Ovine Serum
   7.3.1 Preparation of Tris-HCl and phosphate buffered saline
   7.3.2 Diafiltration of ovine serum
   7.3.3 Centrifugation
   7.3.4 Diethylaminoethyl chromatography
   7.3.5 Biuret assay
   7.3.6 Native gel electrophoresis
   7.3.7 Sodium dodecyl sulphate electrophoresis
   7.3.8 Dialysis of serum fractions
   7.3.9 Biuret assay
   7.3.10 Overview of the protein fractionation procedure

7.4 Wool Follicle Culture in the Presence of Serum Fractions
   7.4.1 Experimental procedure
   7.4.2 Results and discussion

7.5 Discussion and Conclusions
7  **AN EVALUATION OF THE EFFECT OF OVINE SERUM ON WOOL GROWTH AND FOLLICLE VIABILITY IN VITRO**

7.1  **INTRODUCTION**

Attempts at the development of a successful organoid culture technique for fibre follicles have led researchers to incorporate serum into the nutrient medium. Few researchers have evaluated effects of the added serum, on fibre growth. Philpott *et al.* (1990) and Westgate *et al.* (1993) reported inhibitory effects of foetal calf serum when this was supplemented into the culture medium at 1%, 10% or 20%. Frater (1980) evaluated the effect of foetal calf and rat serum on juvenile rat follicles. They found that while juvenile rat and foetal calf serum had no effect, adult rat serum resulted in altered morphology with degeneration of the dermal papilla and trichocyte keratinization. The incorporation of foetal calf serum in the culture medium had no effect on rat (Philpott *et al.* 1989) or mouse follicles (Buhl *et al.* 1989). Supplementation of the tissue culture medium with 1% foetal calf serum also had no effect on the rate of wool growth *in vitro* (Chapter 5). Hibberts and Randall (1992) found that dermal papilla cells isolated from human hair follicles were healthier and grew at a faster rate, when supplemented with human, rather than foetal calf serum. The effect of ovine serum on isolated wool follicles has not been examined but may provide interesting information regarding the regulatory control of wool production.

Blood serum is defined as the fluid expressed from clotted blood or from clotted blood plasma. It is a complex mixture of biomolecules which demonstrate growth-promoting and growth-inhibiting activities. Figure 7-1 outlines the primary components of blood serum and some known functions of these factors. More than 50 minor protein components, including a number of enzymes and growth factors have also been found in blood serum (Lehninger 1975) along with numerous metabolites (Maurer 1986).

Proteins form the largest component of serum. As detailed in Figure 7-1, the non-growth factor peptides include albumin, globulins and complement. Serum albumin has a transport role for lipids, growth factors and minerals. It regulates cellular osmolarity and provides a cellular buffering capacity. Little is known of the actions of α-globulins. The γ-globulins have attached carbohydrate moieties and form the antibody component of serum (Lehninger 1975; Maurer 1986).

The β₁-lipoproteins are very large β-globulin molecules with a molecular weight of approximately 3-20 million. They contain a lipid moiety which makes up about 85% of their molecular weight. Their biological role is one of lipid transport. Transferrin is a further example of the β-globulin family of molecules. Transferrin contains carbohydrate molecules and is required for iron (Fe²⁺) transport (Lehninger 1975; Maurer 1986).
Complement defines a complex system of soluble proteins with about 20 interacting components which are activated during an immune response. Complement proteins have a role in antibody-mediated cell lysis and attract phagocytic cells to sites of infection, enhancing the ability of these cells to ingest and destroy foreign particles. Foetal serum is typically used in tissue culture to avoid any confounding effects due to immune rejection by factors present in adult serum, primarily through complement proteins. However the activity of the complement proteins is rapidly destroyed by heat (Lehninger 1975).

The major roles of serum in vivo are the provision of growth factors for the regulation of cell proliferation, binding proteins for growth factor transport, trace element co-factors and lipids. The removal of waste metabolites and supply of nutrients to peripheral tissues are additional roles of serum. Many co-factors and growth factors are required in trace amounts and only act physiologically when in the bound form (Maurer 1986; Norman et al. 1989). In vitro, serum fulfils these roles, in addition to the provision of attachment and spreading factors which are essential for the establishment of cell lines in tissue culture (Maurer 1986).

The specific hypothesis evaluated here is that ovine serum is capable of providing undefined factors which affect the maintenance and growth of wool follicles in vitro. A preliminary identification of these factors through serum fractionation techniques was also undertaken. Adult ovine serum was used throughout this study.
7.2 **Effects of Adult Ovine Serum on Wool Growth by Follicles Maintained *in Vitro***

### 7.2.1 Experimental Procedure

Venous blood was collected into 10ml Venoject™ tubes free of anticoagulant. Blood was allowed to clot in iced water for approximately 30 minutes and centrifuged at 4000rpm for 10 minutes to separate serum from other blood components. Aliquots of serum were heat inactivated by maintaining 10ml at 57°C for 30 minutes, to destroy the activity of complement proteins. This was designed to prevent development of an immune response by follicles which were isolated from a separate animal to the serum donor.

Wool follicles were isolated by microdissection and maintained in Control Medium B or Control Medium B supplemented with 20% serum or 20% heat inactivated (HI) serum. All follicles maintained with serum were isolated from a different animal from the serum donor.

On all occasions, animals had been maintained at pasture. Any seasonality of serum effects was monitored by supplementing the nutrient medium with serum collected at a different time of the year, which had been stored frozen. The following experiments were undertaken:

- **Experiment one** - effect of Autumn (April) serum on Drysdale follicles collected in Autumn;
- **Experiment two** - effect of Autumn (April) serum on English Leicester follicles collected in Autumn;
- **Experiment three** - effect of Winter (June) and Spring (November) serum on English Leicester follicles collected in Spring; and
- **Experiment four** - effect of Winter (June) and Spring (November) serum on English Leicester follicles collected in Autumn (March).

### 7.2.2 Results and Discussion

The data for experiment one and two are shown in Table 7-1. Follicles isolated from a Drysdale sheep were not significantly affected by the presence of adult ovine serum. This contrasts with the effect of ovine serum on wool follicles isolated from an English Leicester sheep. Under all conditions examined, the rate of wool growth by isolated English Leicester follicles was inhibited by the presence of ovine serum, with up to a 40% reduction in fibre growth being observed. Both Drysdale and English Leicester serum inhibited fibre production by these follicles and heat inactivation of serum did not remove this inhibition. Thus, inhibition it is not due to immune factors such as complement.
Table 7-1 Fibre growth rate and follicle viability for follicles isolated from Drysdale or English Leicester sheep and maintained in Control Medium B or serum supplemented medium. Indications of significance (P values) refer to differences between treatment and control groups within individual experiments by ANOVA. HI = heat inactivated.

<table>
<thead>
<tr>
<th>Experiment one - Drysdale follicles</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± s.e.m.) (μm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Medium B</td>
<td>44</td>
<td>43</td>
<td>366±28</td>
<td>0.015</td>
</tr>
<tr>
<td>Drysdale HI serum</td>
<td>43</td>
<td>42</td>
<td>271±31</td>
<td>0.027</td>
</tr>
<tr>
<td>Drysdale serum</td>
<td>42</td>
<td>42</td>
<td>280±26</td>
<td>0.044</td>
</tr>
<tr>
<td>English Leicester HI serum</td>
<td>43</td>
<td>43</td>
<td>288±24</td>
<td>0.49</td>
</tr>
<tr>
<td>English Leicester serum</td>
<td>40</td>
<td>39</td>
<td>339±32</td>
<td></td>
</tr>
</tbody>
</table>

Table 7-2 Fibre growth rate and follicle viability of English Leicester follicles isolated at different times of the year and supplemented with 20% frozen English Leicester serum. Indications of significance (P values) refer to differences between treatment and control groups within individual experiments by ANOVA.

All follicles examined in Experiments three and four were isolated from an English Leicester sheep. Data from these experiments are in Table 7-2 and show that follicles isolated in Autumn are more susceptible to inhibition by adult ovine serum than follicles isolated in Spring. Serum collected in winter was more effective at inhibiting fibre growth by wool follicles than spring serum, particularly in the case of follicles isolated in spring.
The results demonstrate that there is clearly an inhibitory effect of adult ovine serum on follicle activity and that this effect varies with sheep breed and with time of year. Drysdale serum was more inhibitory than English Leicester serum on wool growth by isolated Drysdale follicles, whereas serum from both sheep breeds significantly inhibited wool growth by isolated English Leicester follicles. This suggests that the regulation of wool production by the two sheep breeds may not be through an identical mechanism. Wool growth by Drysdale sheep occurs at a relatively constant rate throughout the year. In contrast, English Leicester sheep have a highly variable wool growth according to the season (Lincoln 1990). In English Leicester animals, the rate of wool production may be regulated by circulating systemic factors, rather than simply a response to changes in local factors such as skin temperature. Inhibition of English Leicester follicles by Drysdale serum suggests that these factors may be present in Drysdale serum, but that their ability to affect wool production in Drysdale follicles has been curtailed in this breed. This is supported by the observation that breed specific differences in the response of wool follicles to prolactin occurs (Curlewis 1992).

Contrary to the initial expectations of this study, the regulation of wool growth by ovine serum is inhibitory, rather than stimulatory. It may be that wool growth regulation is achieved through the inhibition of wool production during winter via partitioning of nutrients away from wool production and towards synthesis of alternate proteins. At other times of the year, when there is less environmental and nutritional pressure on the animal, inhibition of wool growth is reduced. The work presented here suggests that the mechanism of this seasonal regulation is through changes in circulating factors rather than changes in mechanisms existing within the wool follicle.
7.3 Fractionation of Ovine Serum

Two litres of adult ovine serum was collected in Winter (August), heat inactivated, and stored frozen prior to fractionation. Figure 7-2 shows diagrammatically the steps undertaken to obtain suitable fractions for follicle culture experiments.

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**Figure 7-2** Diagrammatic outline of the procedure used to fractionate ovine serum prior to the supplementation of tissue culture media.

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### 7.3.1 Preparation of Tris-HCl and Phosphate Buffered Saline

Tris-HCl was prepared by dissolving 48.456g of Tris in 2L ddH2O and adjusting the pH to pH 8.0 with 3M HCl (10X Tris). 500ml of 10X Tris was diluted to 5L with ddH2O to prepare 1X Tris.

Phosphate buffered saline was prepared by dissolving 8.766g NaCl (0.15M) and 2.722g KH2PO4 (20mM) in 1L ddH2O. The pH was adjusted to pH 7.2 with 0.1M NaOH.
7.3.2 DIAFILTRATION OF OVINE SERUM

173ml of serum (FRACTION 1) was diafiltered against 3L 20mM Tris, pH 8.0 using a 30,000 molecular weight cut-off Ultrasette™ tangential flow device (Filtron Technology Corp.). The first 150ml of low molecular weight filtrate was collected as FRACTION 2.

7.3.3 CENTRIFUGATION

The high molecular weight fraction resulting from the diafiltration of ovine serum was centrifuged at 10,000rpm for 30 minutes, with the supernatant collected as FRACTION 3. The pH of the supernatant was 8.2.

7.3.4 DIETHYLAMINOETHYL CHROMATOGRAPHY

Diethylaminoethyl (DEAE) chromatography enables the separation of compounds on the basis of molecular charge. A column of DEAE Sephadex Fast Flow anion exchange resin with approximately 500ml bed volume was prepared and maintained at 4°C. The column was washed with 2 volumes of 10X Tris (200mM) and equilibrated with 1X Tris overnight. The flow rate was maintained at approximately 2ml/minute. Following application of the centrifuge supernatant, eluted proteins were stabilised by the addition of NaCl following elution to produce a 0.15M salt solution. Approximately 500ml of eluent was collected prior to the addition of 100mM NaCl to the eluting buffer. Following elution of a further 1.5L, the salt concentration of the eluting buffer was further increased to 1M NaCl. The elution profile is shown below (Figure 7-3).

![Figure 7-3](image-url)  
*Elution profile of large molecular weight serum proteins following DEAE chromatography.*
7.3.5 **Biuret Assay**

The protein content of samples eluted from the DEAE column was analysed using an enzyme-linked immunosorbent assay (ELISA) biuret assay. This assay specifically tests for the presence of peptide bonds, as alkaline copper sulphate (primary component of the biuret reagent) reacts with compounds containing two or more peptide bonds to give a violet-coloured complex. The depth of colour is a measure of the number of peptide bonds present in the sample. Using the ELISA system, biuret reagent is mixed with the protein solution, heated to 37°C for 10 minutes, cooled and sample absorbance at 540nm compared with a prepared standard curve. Analysis of serum fractions 4-11 resulted in the following protein concentrations (Figure 7-4). Identification of predominant protein fractions was attempted on the basis of their affinity binding. This was confirmed by separation of the major protein components by electrophoresis.

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Calculated protein concentration (mg/ml)</th>
<th>Possible identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>9.13</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.05</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.73</td>
<td>β-globulins</td>
</tr>
<tr>
<td>8</td>
<td>8.96</td>
<td>β-globulins</td>
</tr>
<tr>
<td>9</td>
<td>9.36</td>
<td>β-globulins</td>
</tr>
<tr>
<td>10</td>
<td>1.79</td>
<td>albumin</td>
</tr>
<tr>
<td>11</td>
<td>16.60</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 7-4** Calculated protein concentrations in each of the eluant samples using biuret ELISA. Identification of some samples has been attempted.

7.3.6 **Native Gel Electrophoresis**

Separation of proteins using this technique is due to differential protein charges. The net charge on a protein determines its rate of movement in an electric field; the amino acid composition of the protein and pH of the electrophoresis buffer determine the charge on a protein. The protein composition of eluent samples was determined using native gel electrophoresis. Following dilution of suspected γ-globulin and β-globulin fractions to approximately 2mg/ml and suspected albumin fractions to 1mg/ml, non-denatured proteins (native proteins) were applied to the origin of a polyacrylamide gel using a Phast™ gel system. Control rabbit serum (1mg/ml) was also applied. Due to the low protein content and the absence of protein peaks (Figure 7-3) in fraction five, this fraction was not analysed by native gel electrophoresis.

Plate 7-1 shows the results of this analysis. Table 7-3 details the protein composition of each fraction, following densitometric analysis of the electrophoresis gel.
Table 7-3  Approximate composition of serum fractionation samples following densitometry of the gel shown in Plate 7-1.

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Protein Purity (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>γ-globulin</td>
<td>β-globulin</td>
<td>albumin</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>67.8</td>
<td>16.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>48.0</td>
<td>14.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>11.2</td>
<td>4.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Rabbit Serum</td>
<td>12.9</td>
<td>57.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Plate 7-1  Photograph of native gel electrophoresis of protein fractionation samples, showing purple bands where protein is present.

The assignment of specific identities to the protein components was achieved by comparison of the banding pattern of the serum fractions with the known composition and banding pattern of control rabbit serum under identical conditions.

7.3.7  SODIUM DODECYL SULPHATE ELECTROPHORESIS

For this technique, protein samples are treated with sodium dodecyl sulphate (SDS) which denatures the proteins and affects the protein charge. The rate of protein migration along an
EFFECTS OF OVINE SERUM

Electrophoresis gel depends primarily on the mass of the SDS-polypeptide particle, by molecular exclusion through the gel. The electric field primarily supplies the driving force for molecular sieving in a polyacrylamide gel which incorporates SDS.

The protein composition of eluent samples was further confirmed using this electrophoretic technique. Samples were all diluted to approximately 1 mg/ml and applied to the origin of a polyacrylamide gel. A solution of high molecular weight markers (Gibco BRL, 16001-018) was also applied. Due to the low protein content and the absence of protein peaks (Figure 7-3) in fraction five, this fraction was not analysed by SDS gel electrophoresis. Plate 7-2 shows the resultant gel with the standard curve prepared from the data presented in Figure 7-5. Table 7-4 details the sample protein composition following densitometric analysis of the electrophoresis gel.

Plate 7-2 Photograph of SDS gel following separation of protein fractionation samples, showing purple bands where protein is present.
Molecular weight standard curve created using the molecular weight markers shown in Plate 7-2. This curve was used to determine the protein size of the major components of ovine serum fractions.

\[
y = -27.908 \ln(x) + 376.02 \\
R^2 = 0.9982
\]

**Figure 7-5**

**Table 7-4** Approximate composition of serum fractionation samples following densitometry of the SDS gel shown in Plate 7-2.
7.3.8 **DIALYSIS OF SERUM FRACTIONS**

Tris buffer was removed from each sample by dialysis against phosphate buffered saline for 48 hours at 4°C. Spectra/Por™ porous membrane tubing (molecular weight cut-off 12-14,000 kilodaltons, Cat. no. 132680). All dialysed eluant samples were filtered (0.2μm pore size) and stored frozen until used as tissue culture supplements.

7.3.9 **BIURET ASSAY**

The amount of protein in each dialysed samples was determined using an ELISA biuret system as previously described. Protein concentrations were calculated from absorbance at 540nm after comparison with a standard curve (Table 7-5).

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Calculated protein concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>9.5</td>
</tr>
<tr>
<td>5</td>
<td>1.1</td>
</tr>
<tr>
<td>6</td>
<td>0.7</td>
</tr>
<tr>
<td>7</td>
<td>0.9</td>
</tr>
<tr>
<td>8</td>
<td>9.6</td>
</tr>
<tr>
<td>9</td>
<td>9.7</td>
</tr>
<tr>
<td>10</td>
<td>1.8</td>
</tr>
<tr>
<td>11</td>
<td>16.0</td>
</tr>
</tbody>
</table>

Table 7-5 **Protein concentration of each of the eluant samples.**
### 7.3.10 Overview of the Protein Fractionation Procedure

Fractionation of adult ovine serum using the above protocol gave samples differing in their protein composition and purity. The results from this procedure are summarised in Table 7-6 and discussed below.

<table>
<thead>
<tr>
<th>Serum Fraction</th>
<th>Protein Composition</th>
<th>Protein Purity</th>
<th>Protein Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>native globulins</td>
<td>SDS electrophoresis protein size (kilodaltons)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>y-</td>
<td>beta-</td>
<td>&gt;350</td>
</tr>
<tr>
<td>1 serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 salts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 supernatant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 1st sample</td>
<td></td>
<td></td>
<td>37%</td>
</tr>
<tr>
<td>5 2nd sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 3rd sample</td>
<td></td>
<td></td>
<td>26%</td>
</tr>
<tr>
<td>7 4th sample</td>
<td></td>
<td></td>
<td>31%</td>
</tr>
<tr>
<td>8 5th sample</td>
<td>68%</td>
<td>17%</td>
<td></td>
</tr>
<tr>
<td>9 6th sample</td>
<td>48%</td>
<td>14%</td>
<td>15%</td>
</tr>
<tr>
<td>10 7th sample</td>
<td>11%</td>
<td>5%</td>
<td>65%</td>
</tr>
<tr>
<td>11 8th sample</td>
<td></td>
<td></td>
<td>42%</td>
</tr>
</tbody>
</table>

**Table 7-6** Summary of the protein composition and purity of the serum fractions produced in this study.

Fraction one, heat inactivated adult ovine serum, consists of a complex mixture of large molecular weight protein and lipid molecules, in addition to glucose, amino acids and numerous other organic and inorganic components. Fraction two results from dialfiltration of serum using a 30 kilodalton (kD) molecular weight cut-off membrane. Thus, all later fractions contain only molecules greater than 30kD. Fraction two is likely to contain small proteins such as insulin (6kD) and myoglobin (17kD) as well as glucose, amino acids and other small organic and inorganic molecules. Fraction three contains all serum components greater than 30kD. This will include the majority of serum proteins (Lehninger 1975).

Approximately one third of the proteins present in fraction four consist of large molecular weight species (greater than 350kD). On the basis of size, these may be α₁-lipoproteins (200-400kD). A further 41% of the protein in this fraction is approximately 130kD and is of unknown composition. Fractions five, six and seven had a very low final protein concentration. The composition of fractions six and seven are similar. They appear to contain the proteins detailed for fraction four, with the addition of a 260kD species which may also be α₁-lipoproteins.

Almost all of the protein (87%) present in fraction eight is of one type, with a molecular weight of approximately 140kD. These size data support the initial observation from the location of bands on the native gel (Plate 7-1), that this is γ-globulin (150kD). Fraction nine also contains γ-globulin. A 55kD protein species is also present in fraction nine. Once again, the size data
support the identity of this protein being albumin (68kD). Fractions ten and eleven consist primarily of this latter protein, with fraction eleven containing eight times the final protein concentration of fraction ten. Analysis by native gel electrophoresis suggests that fractions eight, nine and ten also contain β-globulin. This could not be confirmed by SDS gel electrophoresis as the major component of this protein group are the β₁-lipoproteins which have a molecular size of 3-20 million kilodaltons. It is unlikely that molecules of this size would move from the origin under the conditions of SDS electrophoresis and therefore would not be detected.

7.4 Wool Follicle Culture in the Presence of Serum Fractions

In earlier sections of this chapter it has been shown that heat inactivated adult ovine serum inhibits wool growth and that this effect is enhanced when serum collected during winter is applied to follicles isolated during winter. An attempt has been made here, to identify which components of serum are responsible for this inhibitory effect on wool growth. The serum fractions collected above were added to the tissue culture medium, to determine which fractions had an inhibitory influence on fibre growth.

7.4.1 Experimental Procedure

The effect of serum fractions one to eleven, on follicles in culture, was evaluated by aseptically supplementing Control Medium B with each solution to a final concentration of 20%. Due to the number of fractions to be examined, experiments were undertaken on ten separate days. In each experiment, one group of follicles was maintained in Control Medium B, and three groups of follicles were maintained in Control Medium B supplemented with one of the serum fractions. Multivariate analysis was used to normalise the growth rate of fibres maintained in Control Medium B. This removed between-day fibre growth rate variations and enabled a direct comparison between follicles maintained in the presence of serum, and those maintained in Control Medium B over the entire study. All follicles were isolated from the same Drysdale sheep during Winter (July).

Nutrient medium dilution effects were examined by maintaining wool follicles in Control Medium B supplemented with 20% physiological saline.

7.4.2 Results and Discussion

There was a reasonably small degree of variation between experiments, with regard to the growth rate of follicles maintained in Control Medium B. The range of fibre growth rate values for follicles maintained in Control Medium B was 229-362 μm per day, with an average of 298±18 μm per day (mean ± S.E.M.).
Table 7-7 The effect on fibre growth and viability, of maintaining isolated wool follicles in Control Medium B, Control Medium B supplemented with one of the serum fractions defined previously, or physiological saline.

<table>
<thead>
<tr>
<th>Serum fractions</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± S.E.M.) (μm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Medium B</td>
<td>266</td>
<td>250</td>
<td>298±18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1 serum</td>
<td>205</td>
<td>200</td>
<td>233±23</td>
<td>0.28</td>
</tr>
<tr>
<td>2 salts</td>
<td>62</td>
<td>56</td>
<td>274±26</td>
<td>0.18</td>
</tr>
<tr>
<td>3 supernatant</td>
<td>25</td>
<td>25</td>
<td>255±35</td>
<td>0.07</td>
</tr>
<tr>
<td>4 1st column fraction</td>
<td>45</td>
<td>42</td>
<td>252±29</td>
<td>0.23</td>
</tr>
<tr>
<td>5 2nd column fraction</td>
<td>19</td>
<td>18</td>
<td>253±39</td>
<td>0.06</td>
</tr>
<tr>
<td>6 3rd column fraction</td>
<td>26</td>
<td>23</td>
<td>232±37</td>
<td>0.07</td>
</tr>
<tr>
<td>7 4th column fraction</td>
<td>53</td>
<td>48</td>
<td>232±29</td>
<td>0.007</td>
</tr>
<tr>
<td>8 5th column fraction</td>
<td>57</td>
<td>49</td>
<td>278±29</td>
<td>0.43</td>
</tr>
<tr>
<td>9 6th column fraction</td>
<td>36</td>
<td>31</td>
<td>227±31</td>
<td>0.01</td>
</tr>
<tr>
<td>10 7th column fraction</td>
<td>67</td>
<td>60</td>
<td>227±26</td>
<td>0.001</td>
</tr>
<tr>
<td>11 8th column fraction</td>
<td>57</td>
<td>48</td>
<td>112±28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Physiological saline</td>
<td>19</td>
<td>19</td>
<td>331±39</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Serum fractions one, seven, nine, ten and eleven significantly inhibited the rate of wool growth by isolated follicles (P<0.01).

Fraction one is complete adult ovine serum (heat inactivated). The results support those presented earlier in this study and confirm that ovine serum collected during winter, inhibits the rate of wool growth of follicles isolated during winter and maintained in vitro. This inhibition is not due to low molecular weight components of serum which are present in fraction two. Fraction three could therefore be expected to contain the components of serum responsible for inhibition of wool growth. However inclusion of fraction three in the nutrient medium did not significantly retard fibre growth (P=0.18). Follicles maintained in the presence of 20% fraction three grew at 85% of the rate of control follicles.

Follicles maintained in the presence of fraction seven grew at 78% of the rate of follicles maintained in Control Medium B. Fraction seven had a relatively low final protein concentration (0.9mg/ml) suggesting that the inhibition of fibre growth may be due to the presence of a powerful inhibitor. The composition of this fraction appears very similar to fraction six and fraction four, both of which also inhibited wool growth (P=0.06 and P=0.07 respectively). The large molecular weight species (possibly α₁-lipoproteins) or the unidentified 130kD protein appear to be responsible for the inhibition.

Inhibition by fractions nine, ten and eleven was highly significant for wool growth (P=0.01, 0.001 and <0.001 respectively). It appears that the major inhibitory protein in these fractions could be albumin.
7.5 DISCUSSION AND CONCLUSIONS

The presence of ovine serum in the nutrient medium has a significant inhibitory effect on wool growth by isolated follicles. This effect is most apparent in follicles isolated during winter and in all follicles supplemented with serum collected during winter. This study also shows differential breed effects in the degree of inhibition elicited by ovine serum. Fibre growth by follicles from English Leicester sheep was more sensitive to inhibition by ovine serum than follicles from Drysdale sheep. It would be interesting to examine the effects of individual serum fractions on English Leicester follicles.

The inhibition of wool growth by adult ovine serum is not due to immunological rejection. Heat inactivation, which reduces the activity of complement proteins, did not affect the ability of serum to inhibit fibre growth. It would be interesting to further investigate this observation by examining the effect on in vitro fibre growth, of serum obtained from the same animal as the wool follicles.

The serum fractionation procedure described in this study has shown that at least two major protein components in serum are responsible for the observed inhibition of wool growth. These have tentatively been defined as one of α₁-lipoprotein or an unidentified 130kD protein, and albumin. The identification of the protein components is on the basis of native and SDS gel electrophoresis analysis results. Considerable work is required to confirm this.

The mechanism by which wool growth inhibition is occurring is not apparent. The molecular size of growth factors is in the region of 21kD (prolactin, growth hormone) to about 3-5kD (insulin, glucagon) (Lehninger 1975), therefore these do not appear to be the regulatory factors described here. Therefore, two potential regulatory mechanisms are that either (a) the proteins identified are having direct effects or (b) that these proteins are acting as binding proteins for trichocyte growth factors. Given that growth factors are typically found in trace amounts and that they are transported, and frequently act, in bound form, the hypothesis that bound growth factors are responsible for these effects is most appealing. If growth factors are responsible, the quandary then exists as to the likely identity of such agent/s. They must be endocrine in action as they are circulated in ovine serum, rather than synthesised at the wool follicle. Endocrine factors which are under direct pituitary control are shown in Figure 7-6.
Removal of the pituitary gland (hypophysectomy) has been shown to reduce wool growth (Wallace 1979). This does not, therefore, provide supporting evidence for a role for the pituitary growth factors in the inhibitory regulation of wool growth demonstrated in this chapter. If a pituitary, or pituitary-controlled growth factor were responsible, removal of the pituitary could be expected to remove the inhibitory regulation, with a consequential increase in wool production. This simplistic finding is, however, confounded by the realisation that, like all such experimentation, hypophysectomy may reduce wool growth through mechanisms not related to normal wool growth regulation in the intact animal. Wool growth is similarly reduced following thyroidectomy and adrenalectomy (Ferguson et al 1965; Wallace 1979).

An opening therefore exists for consideration of the non-pituitary regulated endocrine growth factors. Calcitonin, parathyroid hormone, insulin and glucagon are not directly regulated by anterior pituitary factors. Adrenaline and noradrenaline are further examples of this type of growth factor (Lehninger 1975). Insulin-like growth factor, epidermal growth factor, transforming growth factor and fibroblast growth factor also have the potential to act as protein-bound, endocrine growth factors.
While the mechanisms have not been defined, this study clearly shows that factors exist within sheep serum which inhibit wool growth \textit{in vitro} and it seems likely that similar mechanisms probably occur \textit{in vivo}. The effects of specific growth factors on wool production are evaluated in Chapter's 9 and 10 of this thesis.
CHAPTER EIGHT

AN EVALUATION OF THE EFFECT OF SEASON AND NUTRITIONAL STATUS, PRIOR TO FOLLICLE ISOLATION, ON WOOL PRODUCTION IN VITRO

The true worth of a researcher lies in pursuing what he did not seek in his experiment as well as what he sought.

Claude Bernard (1813-1878)  
French physiologist
8 AN EVALUATION OF THE EFFECT OF SEASON AND NUTRITIONAL STATUS, PRIOR TO FOLLICLE ISOLATION, ON WOOL PRODUCTION IN VITRO

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1 Introduction</td>
<td>115</td>
</tr>
<tr>
<td>8.2 Seasonal Effect</td>
<td>116</td>
</tr>
<tr>
<td>8.2.1 Experimental procedure</td>
<td>116</td>
</tr>
<tr>
<td>8.2.2 Results and discussion</td>
<td>117</td>
</tr>
<tr>
<td>8.3 Nutritional Effect</td>
<td>120</td>
</tr>
<tr>
<td>8.3.1 Experimental procedure</td>
<td>120</td>
</tr>
<tr>
<td>8.3.2 Results and discussion</td>
<td>120</td>
</tr>
<tr>
<td>8.4 Discussion and Conclusions</td>
<td>121</td>
</tr>
</tbody>
</table>
8 \textbf{AN EVALUATION OF THE EFFECT OF SEASON AND NUTRITIONAL STATUS, PRIOR TO FOLLICLE ISOLATION, ON WOOL PRODUCTION \textit{IN VITRO}}

8.1 \textbf{INTRODUCTION}

In the previous chapter, ovine serum was shown to inhibit wool growth \textit{in vitro}. This effect was moderated by sheep breed. For example, the rate of wool growth by follicles isolated from a Drysdale animal was inhibited to a greater degree by Drysdale rather than English Leicester serum. The inhibitory effect of serum was also moderated by the time of year at which follicles and serum were collected. Wool growth by follicles isolated in Autumn was inhibited by serum to a greater extent than follicles isolated in Spring. It was also apparent that wool growth was differentially affected depending on whether the applied serum was collected in Winter or Spring. Winter serum had a greater effect on all follicles irrespective of breed. Identification of the factors in serum that inhibited wool growth was also investigated.

All the findings suggest that the rate of wool growth is regulated by specific factors in serum and that there is a season dependent susceptibility of the follicles to these serum components. Seasonal differences in the composition and/or concentrations of factors in serum may also exist. This is supported by other \textit{in vivo} studies which have shown that there are seasonal differences in the rate of wool growth. In most sheep breeds, wool growth is maximum in summer and minimum in autumn (Coop 1953; Bigham et al. 1978; Geenty et al. 1984; Lincoln 1990). The factors responsible for this cyclic pattern of wool growth have not been conclusively identified. However seasonal fluctuations in photoperiod, temperature and nutrition are implicated (Bennett et al. 1962; Ryder 1978; Allden 1979; Birrell 1992; Coombe 1992).

Regulation of the seasonal variations in wool growth may be through factors originating within the follicle (autocrine or paracrine systems) or via endocrine growth factors. The endocrine growth factors prolactin and melatonin have been implicated as mediators of the photoperiodic response because specific changes in the plasma concentrations of these growth factors occur seasonally. They have also been shown to affect wool follicle activity (Pearson et al. 1993a; Parry et al. 1993), dietary protein intake and nitrogen retention (Curlewls 1992).

In addition to the correlation between wool growth rate and photoperiod, there is a positive relationship between wool growth and feed intake at all times of the year (Coop 1953; Allden 1979; Coombe 1992). For example, consumption of high protein diets increased clean fleece weight (Reis and Downes 1971; Hynd 1989a; Aziz et al. 1991; Bray et al. 1993).

The present work described in this chapter was designed to identify whether factors which regulate the rate of wool growth \textit{in vivo} retain this regulation when follicles are isolated and maintained \textit{in vitro}. Specifically, the hypothesis examined was that seasonal and nutritional modulation \textit{in vivo} result in systemic changes which affect the wool follicle and regulate fibre
production and that these effects are therefore lost in isolated follicles. This was examined by isolating follicles at regular intervals during a ten month period and comparing the rate of wool growth *in vitro* with that occurring *in vivo*. Follicles were also isolated from sheep maintained on two planes of nutrition in order to compare wool growth rates *in vivo* and *in vitro*.

### 8.2 Seasonal Effect

That there are seasonal differences in the rate of wool growth *in vivo* has been reviewed above and in the Section 2.8.1. This study attempts to examine whether this regulation is achieved through systemic factors or by physiological changes in the follicle itself. If such regulation is due to systemic factors then any cyclic wool growth variation by follicles on the sheep would be lost when follicles are isolated and maintained in tissue culture. Alternately, if the regulation is due to a change in the follicle itself then differences in the rate of wool production *in vivo* could be expected to be maintained *in vitro* by isolated follicles.

#### 8.2.1 Experimental Procedure

Four English Leicester ewes were maintained outdoors at pasture in a non-pregnant condition for the duration of this experiment (May 1993-February 1994). These four animals were selected from a group of ten animals on the basis of their seasonal wool growth variation during the previous year (June 1992-February 1993). Animals with the greatest change in wool growth between summer and winter were selected.

At 28 day intervals, a wool sample was collected from the midside of each of the four sheep. The same 100mm x 100mm area was clipped on each occasion. *In vivo* clean wool production (mg/cm²/day) was determined for the preceding 28 day interval by detergent washing of this midside patch sample. The clean wool weight was determined after conditioning in a controlled environment (65% relative humidity, 20°C for 24 hours).

The average fibre length and diameter for each wool sample were also determined. Length measurements were obtained by randomly scattering at least 70 clean wool fibres from each midside patch sample onto clear adhesive tape. The tape was then inverted and adhered to a sheet of black paper which allowed individual fibres to be clearly observed. The length of each fibre was traced using a digitiser to determine the average fibre length. Fibre diameter was measured using an optical fibre diameter analyser (OFDA) (Baxter *et al.* 1992).

At the same time as midside patch wool samples were collected, skin strips were taken from the opposite midside of each animal. Individual follicles were isolated from the skin strips and maintained in Control Medium B. The rate of wool growth *in vitro* was determined by measuring changes in the length of isolated follicles in tissue culture.
### Results and Discussion

*In vivo* wool production by the four English Leicester sheep showed typical seasonal fluctuations. Wool production (mg/cm²/day) underwent a seasonal decline during winter and rose to a peak in summer.

![Figure 8-1](image)  
**Figure 8-1** Seasonal variation in the rate of wool growth *in vivo* by four English Leicester sheep during the period May 1993 to February 1994. Each line indicates wool growth data for an individual sheep.

In contrast to this *in vivo* data, isolated wool follicles collected from each of these sheep and maintained *in vitro* showed no variation in the rate of wool growth between the ten monthly samples (Figure 8-2).

![Figure 8-2](image)  
**Figure 8-2** Growth rate of wool fibres from follicles isolated from four English Leicester ewes and maintained *in vitro*. Each line indicates data from one of the four animals.
As shown in Figure 8-3, there was a low, negative correlation between wool production \textit{in vivo} and that occurring \textit{in vitro} ($R^2 = 0.009$, correlation coefficient = -0.09). Each point on the graph represents the wool growth rate of one animal at one of the 28 day sampling intervals.

Figure 8-3 shows that there was no correlation between the rate at which wool grows \textit{in vivo} with that produced by isolated follicles, indicating that the regulation of wool growth is mediated by systemic factors rather than modification of the follicle itself. In other words, isolation of follicles from the sheep removes the systemic regulation and the pattern of seasonal growth rate variation is lost.

The possibility exists, however, that some seasonal variation in follicle physiology and/or morphology may occur. On two occasions during the winter of 1993 (30 June and 27 July) there was a significant increase in the proportion of isolated follicles which were non-viable, compared to other sampling periods during the year. The average number of non-viable follicles for each month of the experiment was $5.7 \pm 1.9$ (mean $\pm$ S.E.M.), except for 30 June 1993 and 27 July 1993 when the figure increased to 18 ($P < 0.01$) and 8 ($P < 0.05$) respectively. The total number of follicles isolated for organoid culture in each month was $90 \pm 3.4$.

This data suggests that there is a reduction in the viability of cultured follicles when they are isolated from sheep during winter. This observation gives rise to the hypothesis that there is a carryover of \textit{in vivo} regulation, and that a reduction in the number of follicles which are in an active growth phase (anagen) during winter \textit{in vivo}, is partly responsible for the observed
EFFECT OF SEASON AND NUTRITION

Depression in the rate of wool growth. This was evaluated by using wool growth information from the four animals in this study. The following formula was derived to determine whether the reduction in \textit{in vivo} wool production over winter is solely due to the observed reduction in fibre volume (length and diameter) over this period, whilst taking into account changes in the liveweight and body size of these animals over the ten month experimental period. This formula calculated the variable termed 'Corrected Variation'.

\[
\text{Corrected Variation} = \left\{ \frac{WP}{FL \times (\pi (FD/2)^2)} \right\} \left\{ \frac{LWT_1}{LWT_0} \right\}^{2/3}
\]

- \( WP \) = wool production over a 28 day period (g/cm\(^2\)/per day)
- \( FL \) = average fibre length \textit{in vivo} measured over that 28 day period (mm)
- \( FD \) = average diameter of fibres grown \textit{in vivo} over that 28 day period (mm)
- \( LWT_0 \) = animal liveweight at the beginning of the experiment (kg)
- \( LWT_1 \) = animal liveweight at the time of midside patch wool sampling (kg)

A plot of Corrected Variation against time is presented in Figure 8-4. No significant trend was apparent over the course of the experiment (\( P > 0.1 \)). However there was a tendency for the Corrected Variation to be lower during winter. This suggests that changes in fibre volume do not fully account for the observed reduction in wool production during winter. Instead, it is possible that a reduction in the proportion of follicles in anagen occurs during winter. This is supported by Parry et al (1991), who found that wool follicle activity in New Zealand Wiltshire sheep peaked during late summer and autumn, with a reduction in activity during winter.

![Figure 8-4](image_url)

\( R^2 = 0.408 \)

**Figure 8-4** Fitted (—) and observed (—) relationship for the calculated factor 'Corrected Variation' using a 5\textsuperscript{th} order polynomial.
8.3 **Nutritional Effect**

The seasonality experiment discussed in the previous section of this chapter showed that seasonal systemic control is removed when wool follicles are isolated from the sheep. In the following section, this work was extended by evaluating whether changes in the rate of *in vivo* wool growth, achieved by nutritional changes, are preserved in isolated follicles.

### 8.3.1 Experimental Procedure

Seven Drysdale sheep were housed indoors and fed either a control (9.6 MJME/kg DM, 7.11% crude protein) or a high energy, high protein diet (11.6 MJME/kg DM, 13.45% crude protein) for 20 days. Four animals were fed the control diet and three the high energy, high protein diet. Clean wool production *in vivo* (mg/cm²/day) over this treatment period was determined from midside patch samples (100mm x 100mm) collected at the completion of the trial, following detergent scouring of the wool and drying in a controlled environment. Individual wool follicles were isolated by tissue biopsy on day 13 of the treatment period from two animals on each nutritional treatment and maintained in Control Medium B. Wool growth *in vitro* was determined by examining the length change of isolated follicles in tissue culture.

### 8.3.2 Results and Discussion

As shown in Table 8-1, there was a 36% increase (*P* < 0.05) in the amount of wool produced *in vivo* by animals consuming the high energy, high protein diet compared to the animals maintained on the control diet. In contrast, follicles isolated from these animals had an identical rate of wool growth *in vitro* (*P* > 0.1). There was no difference between the two groups in the proportion of follicles which remained viable in culture.

<table>
<thead>
<tr>
<th>Feeding regime</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (μm/day)</th>
<th>fibre production (mg/cm²/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control diet</td>
<td>40</td>
<td>39</td>
<td>495 ± 28</td>
<td>1.09 ± 0.09</td>
</tr>
<tr>
<td>high energy + protein</td>
<td>48</td>
<td>48</td>
<td>492 ± 26</td>
<td>1.48 ± 0.05</td>
</tr>
</tbody>
</table>

Table 8-1 Wool production *in vivo* and wool growth and follicle viability *in vitro*, by sheep fed control or energy and protein supplemented diets.

The data provide additional support for the hypothesis that the regulation of wool growth is via systemic factors rather than modification of the wool follicle or the wool follicle function. In other words, the rate of wool growth was significantly higher in those animals on a higher plane of nutrition, but when the follicles were isolated, there was no difference in the rate of wool production in the isolated follicles. There was also no difference in the proportion of viable follicles, suggesting that the majority of follicles were actively producing wool *in vivo*. 
8.4 **Discussion and Conclusions**

The four English Leicester sheep examined in the seasonal study showed cyclical changes in wool growth *in vivo*, with wool production being the lowest during winter. In contrast, *in vitro* wool growth rate was independent of season. The lack of a relationship suggests that the primary factors regulating wool growth originate outside the follicle, particularly as there was no carryover of growth rate when this influence was removed by follicle isolation.

This significant finding provides evidence that seasonal control of wool growth is regulated by factors external to the wool follicle. These factors may involve hormonal regulation via the endocrine pathway and/or seasonal changes in physiological factors such as blood flow or dermal temperature. The growth factors melatonin and prolactin have been shown to vary in synchrony with changing photoperiod (Lincoln and Ebling 1985; Lincoln 1990) and manipulation of these growth factors has been shown to affect wool production (Pearson *et al.* 1993a,b,c). Further work is required to assess the effects of exogenous melatonin and prolactin on wool follicle productivity. The impact of these growth factors is investigated Chapter 9.

During winter, a significant reduction was observed in the viability of follicles during maintenance *in vitro*. A possible explanation is that fewer follicles are active over the winter period, and this is reflected in the random sampling of follicles. To test this hypothesis, effects due to changing wool volume and increasing animal size over the course of the experiment were isolated from total measured wool production in the midside patch using the formula described as Corrected Variation. It appears that the observed seasonal variation was accounted for by these factors. However there remained a slight, non significant trend towards an additional unexplained reduction in wool production during winter. This implies that although there was no significant reduction in follicle activity or density *in vivo* during winter, these factors may have some involvement in the reduced wool growth observed at this time. This hypothesis is supported by recent findings on the effects of changing photoperiod on wool follicle activity. Parry *et al.* (1991) found that wool follicle activity in New Zealand Wiltshire sheep was low in winter, compared with other seasons. In future work it would be useful to identify the growth phase of isolated follicles, prior to placing them in culture.

Animals fed a high protein diet showed an increase in wool production *in vivo*. This effect has previously been reported (Hynd 1989a; Coombe 1992; Bray *et al.* 1993), however the exact mechanism has not been identified. Nutrition affects fibre diameter and growth rate (Allden 1979). In addition, some evidence suggests an effect of nutrition on follicle activity. Follicles isolated from animals receiving different protein diets had identical *in vitro* wool growth rates. Therefore the rate of wool production in isolated follicles was independent of host animal nutrition. This implies that systemic factors regulate the wool growth response to nutrition *in vivo* via a transient mechanism. Such regulation may involve control of the rate of blood flow or nutrient uptake by follicles. Alternatively, a high protein diet may increase the availability of amino acids for keratin synthesis or modify hormonal signals. Nevertheless, factors responsible for the increase in wool production *in vivo* were not present in isolated follicles.
wool growth is affected only while the stimulus is present and there is no long-term modification of the follicle.

In summary, the data are consistent with other studies showing that changes in season and nutrient availability produce extrafollicular changes which affect the wool follicle and alter fibre production in vivo. Follicles isolated from the animal are no longer affected by this regulation. Other results provide some supporting evidence for a reduction in in vivo wool follicle activity during winter and raise the possibility that this may contribute to the reduced rate of wool production over the winter period.
CHAPTER NINE

AN EVALUATION OF THE EFFECT OF MELATONIN AND PROLACTIN ON WOOL PRODUCTION

IN VITRO

No amount of experimentation can ever prove me right; a single experiment can prove me wrong.

Albert Einstein (1879-1955)
9 AN EVALUATION OF THE EFFECT OF MELATONIN AND PROLACTIN ON WOOL PRODUCTION IN VITRO

9.1 Introduction 124
9.2 Experimental Procedure 125
9.3 Results 125
9.4 Discussion and Conclusions 127
9 AN EVALUATION OF THE EFFECT OF MELATONIN AND PROLACTIN ON WOOL PRODUCTION IN VITRO

9.1 INTRODUCTION

The seasonal pelage changes observed in many mammals, including wild sheep and primitive domesticated breeds, includes shedding of the fleece. Even in ‘modern’ breeds, seasonal wool growth occurs, with a peak in summer and a nadir during winter (Coop 1953; Bigham et al. 1978; Geenty et al. 1984; Lincoln 1990). As discussed in the previous chapter, these fluctuations are correlated with changes in photoperiod, temperature and nutrition (Bennett et al. 1962; Ryder 1978; Allden 1979). However seasonal wool growth responses have been shown to vary with sheep breed (Slee 1965; Ryder and Stephenson 1968; Lincoln 1990; Curlewis 1992). Merino sheep are least influenced by photoperiod, while coarser and longwool breeds such as English Leicester, Drysdale and Romney are most influenced (Lincoln 1990).

In the previous chapter, data were presented which showed that the seasonal variation of wool growth occurring in vivo is lost when follicles are isolated and maintained in vitro. This suggests that wool growth regulation does not occur so much through changes in the follicle itself, but rather via systemic effects. Wool growth regulation is therefore likely to be mediated through hormonal factors which may act directly on the follicle or alter physiological phenomena such as blood flow and skin temperature. Assessment of these systemic regulators of seasonal wool growth changes is attempted here.

In vivo, the translation of seasonal photoperiod signals into systemic changes is thought to involve melatonin (Lincoln and Ebling 1985; Bubenik et al. 1986; Mitchell et al. 1991), and the pituitary growth factor prolactin (Allain et al. 1986; Lincoln 1990; Kelly et al. 1991; Badura and Goldman 1992). Melatonin is involved in the regulation of prolactin by inhibiting its synthesis in the anterior pituitary. Intravenous administration of melatonin suppresses prolactin secretion (Martinet et al. 1981; Morgan 1990; Foldes et al. 1990; Badura and Goldman 1992). In sheep, melatonin synthesis occurs during the hours of darkness, with serum melatonin being elevated for a period directly proportional to the length of the night (Morgan 1990). Consequently, serum prolactin concentrations also fluctuate seasonally, with a peak in summer and a nadir in winter (Lincoln and Ebling 1985; Lincoln 1990); a pattern synchronous with that of wool production in vivo.

In sheep breeds which undergo a photoperiod-induced moult, reduced circulating prolactin due to a decrease in daylength (natural or manipulated) or melatonin supplementation, occurs in concert with changes in wool production. These changes manifest as production of the winter coat (Allain et al. 1986; Lincoln 1990; Parry et al. 1993), maintenance of high follicle activity (Pearson et al. 1993a) and a reduction in food intake (Lincoln and Ebling 1985; Lynch and Russel 1990). Conversely, a sustained increase in serum prolactin in these breeds, due to increasing daylength or suppression of melatonin, occurs in conjunction with production of a
summer coat and spring moult (Lincoln 1990). A decrease in the proportion of active wool follicles (Pearson et al. 1993a; Parry et al. 1993; Craven et al. 1994) and an increase in dietary protein intake and nitrogen retention (reviewed by Curlewis 1992) have also been observed under these conditions.

Given that manipulation of serum melatonin and prolactin affect wool production in specific sheep breeds (Pearson et al. 1993a), and prolactin binding sites have been identified in wool follicles of Romney, Merino and New Zealand Wiltshire sheep (Choy et al. 1995), it seems likely that these growth factors, and specifically prolactin, directly affect the wool follicle. It is not known whether these and other systemic growth factors regulate changes in follicle output by the action of extrafollicular agents or whether local skin or follicle agents are involved.

The work presented here examines the hypothesis that melatonin and prolactin affect the rate of wool production by isolated follicles. This will enable evaluation of whether these growth factors have a role in the systemic mediation of seasonal wool growth regulation. This hypothesis was examined by measuring the rate of wool growth by isolated follicles in the presence or absence of melatonin or prolactin.

9.2 Experimental Procedure

Wool follicles were isolated following tissue biopsy from the midside of one Drysdale, one English Leicester and one New Zealand Wiltshire. All were adult animals and had all been maintained at pasture during Autumn (April and May).

Three experiments were undertaken to evaluate the effects of prolactin and melatonin on follicles isolated from each of the sheep breeds. In Experiment one, the effect of prolactin concentration was examined by maintaining follicles isolated from the English Leicester sheep in Control Medium B or in Control Medium B supplemented with prolactin (10, 140, 1000ng/ml). In Experiment two, the effect of the three different concentrations of prolactin was evaluated in an alternate sheep breed by repeating Experiment one using follicles isolated from a New Zealand Wiltshire sheep. In the final experiment, breed effects were further evaluated by maintaining follicles isolated from the Drysdale and English Leicester animals in Control Medium B or Control Medium B supplemented with 600ng/ml prolactin or 1.2nM melatonin. Prolactin (ovine, L-6520, 31 i.u./mg) and melatonin (M-5250) were supplied by Sigma Chemicals (St Louis, USA).

In each experiment the rate of wool growth and follicle viability for a particular treatment group of follicles was considered relative to that of follicles maintained in Control Medium B for that particular experiment.

9.3 Results

The presence of prolactin in the tissue culture medium had no effect on the rate of wool growth from isolated English Leicester follicles. Those data are in Table 9-1. None of the three concentrations of prolactin affected follicle viability in vitro.
**Table 9-1** Wool growth by English Leicester follicles in vitro maintained without prolactin (Control Medium B) or with various prolactin concentrations.

<table>
<thead>
<tr>
<th>Prolactin Concentration</th>
<th>Number of follicles isolated</th>
<th>Number of follicles viable</th>
<th>Fibre growth rate (mean ± S.E.M.) (µm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Medium B</td>
<td>24</td>
<td>22</td>
<td>202 ± 32</td>
<td></td>
</tr>
<tr>
<td>10 ng/ml prolactin</td>
<td>24</td>
<td>23</td>
<td>205 ± 23</td>
<td>0.93</td>
</tr>
<tr>
<td>140 ng/ml prolactin</td>
<td>22</td>
<td>21</td>
<td>207 ± 35</td>
<td>0.92</td>
</tr>
<tr>
<td>1000 ng/ml prolactin</td>
<td>23</td>
<td>21</td>
<td>257 ± 31</td>
<td>0.23</td>
</tr>
</tbody>
</table>

There was also no effect of up to 1000ng/ml prolactin on the viability and growth rate of New Zealand Wiltshire follicles maintained in vitro, as shown by the data in Table 9-2. The rate of wool production in vitro was similar for follicles isolated from English Leicester and New Zealand Wiltshire sheep in this study.

**Table 9-2** Fibre growth by follicles isolated from New Zealand Wiltshire sheep and maintained in vitro without supplements (Control Medium B), or with various concentrations of prolactin in the medium.

<table>
<thead>
<tr>
<th>Prolactin Concentration</th>
<th>Number of follicles isolated</th>
<th>Number of follicles viable</th>
<th>Fibre growth rate (mean ± S.E.M.) (µm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Medium B</td>
<td>22</td>
<td>21</td>
<td>264 ± 22</td>
<td></td>
</tr>
<tr>
<td>10 ng/ml prolactin</td>
<td>24</td>
<td>23</td>
<td>219 ± 26</td>
<td>0.24</td>
</tr>
<tr>
<td>140 ng/ml prolactin</td>
<td>24</td>
<td>24</td>
<td>278 ± 28</td>
<td>0.60</td>
</tr>
<tr>
<td>1000 ng/ml prolactin</td>
<td>23</td>
<td>23</td>
<td>244 ± 25</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Data obtained from Experiment three (Table 9-3) show that there was also no effect on the rate of fibre growth or follicle viability of either prolactin or melatonin on Drysdale or English Leicester follicles.
**Table 9-3**  
Fibre growth by follicles isolated from Drysdale or English Leicester sheep maintained *in vitro* without supplements (Control Medium B), or in medium supplemented with 1.2nM melatonin or 600ng/ml prolactin. Differences in the proportion of viable follicles were determined by \( \chi^2 \) analysis; \( ** \) \( P<0.01 \).

<table>
<thead>
<tr>
<th>Experiment three</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± s.e.m.) (μm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drysdale follicles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Medium B</td>
<td>35</td>
<td>34</td>
<td>394 ± 25</td>
<td>0.59</td>
</tr>
<tr>
<td>600ng/ml prolactin</td>
<td>28</td>
<td>26</td>
<td>374 ± 28</td>
<td>0.56</td>
</tr>
<tr>
<td>1.2nM melatonin</td>
<td>27</td>
<td>26</td>
<td>416 ± 28</td>
<td></td>
</tr>
<tr>
<td><strong>English Leicester follicles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Medium B</td>
<td>39</td>
<td>36</td>
<td>246 ± 23</td>
<td></td>
</tr>
<tr>
<td>600ng/ml prolactin</td>
<td>28</td>
<td>22**</td>
<td>263 ± 26</td>
<td>0.63</td>
</tr>
<tr>
<td>1.2nM melatonin</td>
<td>39</td>
<td>36</td>
<td>256 ± 27</td>
<td>0.79</td>
</tr>
</tbody>
</table>

As noted in Table 9-3, the fibre growth rate in Control Medium B was significantly higher for Drysdale follicles than for those isolated from an English Leicester sheep (\( P<0.01 \)). This concurs with previous findings of this study (Chapter 7) where it was determined that the rate of wool growth *in vitro* by follicles isolated from Drysdale sheep exceeded that by follicles from English Leicester.

From the three experiments it is apparent that supplementation of the basal culture medium with prolactin or melatonin had no significant effect on the rate of fibre extension from follicles isolated from Drysdale or English Leicester animals. Similarly, prolactin supplementation had no significant effect on the *in vitro* fibre growth rate of follicles isolated from New Zealand Wiltshire sheep. The presence of prolactin in the culture medium had no significant effect on the viability of wool follicles from any sheep breed examined. One exception was English Leicester follicles exposed to 600 ng/ml prolactin (Table 9-3) which showed a significant reduction in follicle viability (21% non-viable vs 7% non-viable; \( P<0.01 \)). This is likely to be a chance result given that double the prolactin concentration had no effect.

### 9.4 Discussion and Conclusions

The range of sheep plasma prolactin concentrations is 0 - 200 ng/ml depending on breed and time of year (Lincoln 1990; McCloghry *et al.* 1992b). Isolated wool follicles subjected to five times the physiological concentrations of plasma prolactin had no effects on the growth rate or viability of the wool follicles. The concentration of plasma melatonin in sheep ranges from 0 - 400 pM depending on the time of day, season and possibly sheep breed (Morgan 1990; Lincoln 1990). In this study, exposing follicles to three times the maximum physiological concentration of melatonin had no effect on fibre growth rates.
One possible reason why wool follicles do not respond to prolactin or melatonin in this work is that the selected sheep breeds themselves are not responsive to the growth factors. However this hypothesis would not appear to hold for the New Zealand Wiltshire sheep since work by Pearson et al. (1993a,b) indicates that this breed undergoes a moult which is apparently mediated by increased plasma prolactin concentrations. Alternatively, it may be that the culture conditions were not optimised to produce in vitro follicle responses to these growth factors. These two possibilities are evaluated below.

The hypothesis that wool follicles are unaffected by prolactin or melatonin requires further evaluation of current evidence. For example, serum prolactin concentrations have been shown to fluctuate seasonally in a pattern synchronous with that of wool production in vivo (Lincoln and Ebling 1985; Lincoln 1990). In addition, manipulation of serum prolactin concentrations does affect wool production in vivo (Allain et al. 1986; Lincoln 1990; Parry et al. 1993). The identification of prolactin binding sites in the wool follicles of Romney, Merino and New Zealand Wiltshire sheep (Choy et al. 1995) also provides additional evidence that prolactin acts directly in the regulation of pelage. Nonetheless, effects of prolactin and seasonal changes, on wool production, is dependent on the sheep breed under examination. For example, in the Wiltshire Horn, Mouflon and Soay breeds, a seasonal increase in plasma prolactin concentration is typically correlated with an increased proportion of follicles in anagen (active growth phase), a resurgence of wool growth and a conspicuous moult (Ryder 1971; Lincoln 1990). However, in other breeds such as the Romney and Merino, no clear seasonal shedding is observed and wool growth continues throughout the year (Lincoln and Ebling 1985; Lincoln 1990; McCloghry et al. 1992a,b). Although these breed differences in the response to photoperiod exist, all breeds continue to demonstrate a clearly defined cycle of prolactin secretion (Curlewis 1992; Clarke et al. 1993).

In shedding sheep breeds such as the New Zealand Wiltshire, manipulation of plasma prolactin levels alters wool production. In these sheep, a sudden increase in photoperiod, with a consequential increase in plasma prolactin, results in shedding of the fleece (Pearson et al. 1993b), whilst suppression of the prolactin surge with bromocryptine results in maintenance of the follicle activity and no fleece shedding occurs (Pearson et al. 1993a). In contrast, in highly developed wool bearing sheep, manipulation of plasma prolactin failed to change wool production (Wallace 1979). Indeed, complete suppression of prolactin in Romney (McCloghry et al. 1992b; McCloghry et al. 1993; Clarke et al. 1993) and Merino sheep (Foldes et al. 1990) had no effect on fleece growth rate.

It therefore appears that in some sheep breeds, the seasonal cycles of pelage growth have been modified by selective breeding for high quality fleeces. An altered response of the wool follicle to photoperiod may result from factors such as changes in the synthesis or release of prolactin from the anterior pituitary or to changes in the number or sensitivity of prolactin receptors in the wool follicle (Lincoln 1990; McCloghry et al. 1992a; Curlewis 1992).
In cashmere goats, increased prolactin due to domperidone administration, reduced fibre growth in primary but not secondary follicles (Litherland and O’Neill 1994). In contrast, secondary hair follicles from a cashmere goat maintained in vitro responded to 200ng/ml supplementation of the nutrient medium with elongation of the hair shaft with predominant effects occurring in the first two days of culture (Ibraheem et al. 1993a,b).

The duration of the wool growth cycle also appears to have been altered by selection. For example, Soay wool follicles remain in anagen for approximately six months whereas Merino wool follicles continue active growth for up to eight years (Ryder and Stephenson 1968; Ryder 1971). This change in the follicle growth cycle may be involved in the altered response to prolactin in highly developed wool growing breeds.

A second possible cause for the lack of an in vitro effect of prolactin and melatonin on wool growth is that the maintenance of isolated wool follicles for four days is insufficient for changes in wool growth to be recorded. Supporting evidence is that in vivo experiments with New Zealand Wiltshire sheep showed that although changes in the plasma prolactin concentration occur within 7 days, consequential changes in wool growth were only observed after three weeks (Pearson et al. 1993b). This result suggests that maintenance of follicles in vitro for four days may be insufficient to induce observable wool growth effects. In contrast, this duration of culture has been sufficient to demonstrate growth factor effects in other species. For example, isolated hair follicles from cashmere-bearing goats (Ibraheem et al. 1993b; Ibraheem et al. 1994) and red deer (Thomas et al. 1993) exposed to prolactin for five days showed an increase in hair growth. Isolated follicles from cashmere-bearing goats also exhibited an increase in fibre growth during five days in the presence of melatonin (Ibraheem et al. 1994). It is not apparent why melatonin should affect follicles directly or why both melatonin and prolactin should increase wool growth in vitro yet have a contrasting effect in vivo. These results infer that fibre growth is under a very complex endocrine control which is breed dependent.

In summary, the data in this chapter are at variance with the hypothesis that melatonin or prolactin are involved in the mediation of seasonal wool growth at the follicle level in the sheep breeds examined. It has been proposed that these results reflect the breeds examined since there is evidence that the in vivo responses to these endocrine mediators of photoperiod are breed dependent. It is also concluded that the suppression of follicle responses to prolactin and melatonin is due to genetic selection that has occurred during the domestication of sheep and the requirement for continued wool growth rather than seasonal wool shedding.

Development of in situ RT-PCR (reverse transcriptase polymerase chain reaction) and in situ hybridisation techniques would allow further evaluation of this hypothesis. These techniques would allow changes in the site and rate of gene expression to be evaluated following a growth factor challenge.
The scientist values research by the size of its contribution to that huge, logically articulated structure of ideas which is already, though not yet half built, the most glorious accomplishment of mankind.

Sir Peter B. Medawar (1915-1987)
in 'The Art of the Soluble'
10 An Evaluation of the Effect of Potential Growth Affecting Agents on Wool Production In Vitro

10.1 Introduction

10.2 Insulin
- 10.2.1 Experimental procedure
- 10.2.2 Results and discussion

10.3 Insulin-Like Growth Factor-I
- 10.3.1 Experimental procedure
- 10.3.2 Results and discussion

10.4 Fibroblast Growth Factor
- 10.4.1 Experimental procedure
- 10.4.2 Results and discussion

10.5 Epidermal Growth Factor
- 10.5.1 Experimental procedure
- 10.5.2 Results and discussion

10.6 Transforming Growth Factor alpha
- 10.6.1 Experimental procedure
- 10.6.2 Results and discussion

10.7 Ethanolamine / Phosphoethanolamine
- 10.7.1 Experimental procedure
- 10.7.2 Results and discussion

10.8 Minoxidil
- 10.8.1 Experimental procedure
- 10.8.2 Results and discussion
10.9 Antler Extract

10.9.1 Experimental procedure
10.9.2 Results and discussion

10.10 Discussion and Conclusions
10 AN EVALUATION OF THE EFFECT OF POTENTIAL GROWTH AFFECTING AGENTS ON WOOL PRODUCTION IN VITRO

10.1 INTRODUCTION

The development of a serum free follicle culture model has been detailed and evaluated in the preceding chapters of this study. In Chapter 9, mitogenic effects of the potential mediators of photoperiod, melatonin and prolactin, on fibre growth were evaluated using isolated wool follicles. This study now focuses on additional factors which have the potential to regulate fibre growth.

It is likely that a number of growth factors are required for the sustained maintenance of wool production and that the activity of these growth factors is interrelated. This is supported by the observation in the current study that isolated wool follicles remain viable for only a limited duration in the absence of supplementary growth factors and by in vivo studies which have shown that removal of the pituitary (Wallace 1979; Tsao et al. 1982) or thyroid glands (Wallace 1979; Maurer 1986; Donald et al. 1994) perturb wool production. It has also been demonstrated that while many fleece characteristics are determined genetically (Nagorcka 1979; Allden 1979; Reis 1988; Marshall and Gillespie 1989), wool growth is markedly growth factor-dependent (Ferguson et al. 1965; Downes and Wallace 1965; Wallace 1979; Johnsson et al. 1985; Sun et al. 1992; Chaidarun et al. 1994). An animal’s genetic potential is limited by growth factor mediated physiological interactions of nutrition, reproductive status, climate and disease (Wallace 1979; Robards 1979; Hynd 1989b).

The development of a tissue culture model now enables effects of potential mitogens on fibre production to be evaluated in an environment isolated from confounding systemic events. This can be achieved by supplementing the nutrient medium with potential growth factors. While the replacement of individual growth factors in the follicle culture system may not reflect all the in vivo growth factor actions, the in vitro model does enable any direct effects of the growth factors on wool follicle activity to be determined.

Supplementation of the culture medium with growth factors first requires the identification of the concentration at which the growth factors affect a measurable parameter. Furthermore, the active concentration of an exogenously supplied growth agent may not correlate with the physiological concentration because of the tissue culture environment. Typically, higher than normal physiological concentrations are required for in vitro experiments in order to overcome factors such as the limited growth factor half-life, lack of appropriate binding proteins or unsuitable co-factor ionic concentrations (Fischer et al. 1980; Maurer 1986; Breidahl et al. 1989). Each of these may affect growth factor activity and/or receptor binding kinetics. In addition, growth factors can have a stimulatory or a depressive effect on the same process in vitro, depending on the applied concentration (Downes and Wallace 1965; Philpott et al. 1990). These findings suggest that the identification of hormonal effects on fibre growth in vitro requires
dose-response studies. This approach will be undertaken for the mitogens evaluated in this study unless specifically indicated.

Endocrine regulation of wool growth is likely to be achieved through interaction of systemic growth factors with receptors on the surface of cells in the wool follicle, possibly within the dermal papilla of the follicle bulb. Studies by Reynolds and Jahoda (1991a) have established that the dermal papilla plays an important role in hair follicle initiation and maintenance and in fibre formation. This role is likely to be initiated by circulating systemic growth factors which stimulate the secretion of regulatory substances by dermal papilla cells which in turn alter the activity of other follicular cell types and thereby hair growth (Randall et al. 1992). There are several possible mechanisms by which this regulation may be translated into wool growth. For example, the systemic growth factor status may regulate the rate of cell division in the proliferative zone of the follicle bulb, the growth of cells which have moved beyond this zone or the mobilisation of specific limiting nutrients which in turn control the rate of synthetic activity in the follicle (Ferguson et al. 1965).

The growth factors insulin, insulin-like growth factor-I, fibroblast growth factor, epidermal growth factor and transforming growth factor alpha are growth factor candidates for regulating wool growth because of their biological importance in vivo and their effects on cell maintenance in vitro (Fischer et al. 1980; Maurer 1986; Nixon et al. 1994b). The specific potential effects of these growth factors were detailed in Chapter 2. In addition, Minoxidil, the active component of the commercially available hair restorative product Regain™, has been shown to affect human hair growth in vitro (Baden et al. 1988). The effects of Minoxidil on wool growth were examined in this chapter. An aqueous extract of deer antler was also evaluated following the observation that the extract affected events of antler cells in vitro (Suttie et al. 1994). Lastly, a combination of ethanolamine and phosphoethanolamine was added to the nutrient medium to identify whether these pituitary factors affected wool growth, particularly since ethanolamine and phosphoethanolamine have been reported to have potent stimulatory effects on the growth of human keratinocyte growth in vitro (Hawley-Nelson et al. 1980). Each of these growth factors are discussed in detail below and their effects on the activity of wool follicles evaluated.

10.2 INSULIN

Insulin is an endocrine polypeptide growth factor which is synthesised and released from the pancreas in response to a high concentration of blood glucose (Sainz et al., 1990). Activation of the insulin receptor on peripheral tissues can stimulate glycogen synthetase, inhibit lipolysis, promote cellular glucose uptake or stimulate protein synthesis. All these metabolic pathways enhance the conversion of blood glucose to glycogen and lipids and/or increase protein synthesis (Lehninger 1975). While a role for insulin in the regulation of wool production has not been supported by in vivo studies (Wynn et al. 1988; Bray et al. 1990), the cellular responses to insulin infer that this growth factor may have the potential to regulate wool growth.

Under the experimental conditions of tissue culture, insulin rapidly loses its bioactivity because of its short half life and sensitivity to inactivation by cysteine in serum free media (Maurer 1986).
Tissue culture media are, therefore, typically supplemented with a relatively high concentration of insulin (1-10μg/ml) which is considerably higher than physiological levels (0.4-1.6ng/ml in man; Lehninger 1975). Whilst most tissue culture systems include insulin (Philpott et al. 1989; Philpott et al. 1990), further work is required to identify the usefulness and mode of action of insulin. To date, conflicting findings have resulted from insulin supplemented culture systems (Fischer et al. 1980; Maurer 1986; Wilkinson et al. 1987; Breidahl et al. 1989; Norman et al. 1989).

In the study presented here, the effects of insulin on wool fibre growth and follicle maintenance were examined by supplementing the nutrient medium with 0.2ng/ml - 10μg/ml insulin.

10.2.1 EXPERIMENTAL PROCEDURE

Wool follicles were isolated from one Romney sheep and maintained in Control Medium B (which by definition includes insulin supplementation at 10μg/ml) or in William's Medium E supplemented with hydrocortisone, sodium selenite and transferrin and one of four concentrations of insulin (0, 0.2ng/ml, 2ng/ml, 20ng/ml; Sigma Chemical Co.). The length growth rate of wool fibres and viability of isolated follicles was determined by image analysis. Analysis of variance was used to determine the significance of wool growth rate differences between each specific treatment and all other follicles.

10.2.2 RESULTS AND DISCUSSION

The degree of supplementation of the nutrient medium with insulin had no significant effect on wool growth (Table 10-1). Even the complete removal of insulin from the culture medium (insulin-free compared with 10μg/ml insulin) did not affect the rate of wool growth. However, a reduction in the viability of cultured follicles was observed. Ninety percent of follicles remained viable across all treatments, however in the insulin free medium, only 70% of the follicles remained viable (P<0.05).

<table>
<thead>
<tr>
<th>Insulin concentration</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± s.e.m.) (μm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin-free</td>
<td>24</td>
<td>16*</td>
<td>196±30</td>
<td>0.98</td>
</tr>
<tr>
<td>0.2 ng/ml</td>
<td>24</td>
<td>23</td>
<td>191±23</td>
<td>0.79</td>
</tr>
<tr>
<td>2ng/ml</td>
<td>24</td>
<td>23</td>
<td>230±25</td>
<td>0.13</td>
</tr>
<tr>
<td>20ng/ml</td>
<td>24</td>
<td>18</td>
<td>165±23</td>
<td>0.17</td>
</tr>
<tr>
<td>10μg/ml</td>
<td>24</td>
<td>22</td>
<td>193±27</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Table 10-1 Fibre growth by follicles maintained in insulin free medium or in medium supplemented with various concentrations of insulin. Control Medium B is supplemented with 10μg/ml insulin. Differences in the proportion of viable follicles were estimated using χ² analysis; * P<0.05.
EFFECT OF VARIOUS GROWTH FACTORS

These data indicate that insulin is not necessary for the growth of isolated follicles in tissue culture. This supports previous in vivo evidence where it has been shown that wool growth responses can be independent of insulin responses (Bray et al. 1990) and that elevated plasma insulin concentrations may correlate with reduced (Wynn et al. 1988) or increased (Johnsson et al. 1985) wool production. It is possible that the maintenance of follicles over a longer period of time may result in an insulin response, or that higher insulin concentrations are required to elicit an effect in vitro.

There is an indication, however, that in the absence of insulin, there is a reduction in the proportion of follicles which remain viable. Insulin may therefore have a role in maintaining wool follicles in the active, anagen phase of growth and consequently in controlling their regression into a non-active, catagen state. Westgate et al. (1993) found that follicle morphology was better when hair follicles were maintained in vitro in the presence of insulin. This supports the hypothesis that, in the absence of insulin, fibre follicles regress into catagen. Both Westgate et al. (1993) and Bond et al. (1992) reported that the rate of fibre growth was reduced in the absence of insulin. This contrasts with the findings of the current study. It may be that the reported reduction in fibre growth is spurious, in that follicles which became non-viable (entered catagen) in the absence of insulin were included in the growth rate per day calculations. This would result in a low growth rate value for follicles maintained without insulin, but would not truly reflect the rate of wool production by viable follicles.

The present findings therefore indicate that while insulin does not have a major role in the regulation of wool production by follicles in anagen, it may have a role in the regulation of the wool follicle growth cycle. The mechanisms by which the follicle cycle may be regulated by this growth factor requires further investigation.

10.3 INSULIN-LIKE GROWTH FACTOR-I

The insulin-like growth factors (IGFs) were first identified by their ability to promote insulin-like activities. Of the two IGF forms, IGF-I has been most documented regarding potential epidermal effects. While this growth factor is synthesised and secreted primarily by the liver in response to elevated growth factor concentrations (Salmon and Daughaday 1957; Dickson et al. 1991), IGF-I synthesis in dermal fibroblasts has been observed (Sutton et al. 1995). Skin keratinocytes have also been shown to possess IGF-I receptors and to respond to IGF-I (Murashita et al. 1993).

The role of IGFs in the hair follicle is not known, however IGF-I receptors have been identified in the wool follicles of New Zealand Wiltshire sheep. High receptor numbers were identified in the germinat matrix and dermal papilla cells (Nixon et al. 1994a; Nixon et al. 1995a). Targeted mutagenesis of the IGF-I receptor has resulted in abnormal hair follicle development (Krueger et al. 1993). In wool follicles, upregulation of IGF receptor numbers occurs following a hormonal stimulus (Nixon et al. 1995a). This effect is observed prior to any growth effects, therefore this growth factor may have a regulatory role in wool production. However no direct effects of IGF-I have been found on replicating cell numbers in the bulbs of wool follicles (Harris et al. 1993).
Furthermore, infusion of IGF-I in sheep increased skin blood flow, uptake of amino acids (Harris et al. 1993), glucose clearance and protein synthesis but reduced protein catabolism (Breier et al. 1993). There was no reported effect on wool synthesis. However IGF-I has been reported as a potent in vitro stimulator of human hair growth at physiological concentrations (10-100ng/ml) (Philpott et al. 1994).

In Merino sheep, the plasma concentration of IGF-I is 400ng/ml (Magnan et al. 1995). In this study the effects of 3-1000ng/ml IGF-I on the rate of wool growth and follicle viability will be examined using isolated wool follicles.

### 10.3.1 Experimental Procedure

Isolated wool follicles from a Drysdale sheep were maintained in Control Medium B or Control Medium B supplemented with 3, 30, 300 or 1000ng/ml IGF-I. Two separate experiments were undertaken on consecutive days, the data being normalised with respect to fibre growth rates in Control Medium B. The IGF-I was synthesised by Auspep Pty Ltd, Victoria, Australia (Cat.No. 2290) and consisted of amino acids 24-41. The lyophilised powder was reconstituted with Control Medium B.

### 10.3.2 Results and Discussion

The inclusion of IGF-I in the tissue culture medium had no effect on the rate of wool growth or the viability of isolated follicles. The data are presented in Table 10-2.

<table>
<thead>
<tr>
<th>IGF-I concentration</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± S.E.M.) (μm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Medium B (no IGF)</td>
<td>45</td>
<td>39</td>
<td>298±18</td>
<td>0.14</td>
</tr>
<tr>
<td>3ng/ml</td>
<td>16</td>
<td>14</td>
<td>236±44</td>
<td>0.69</td>
</tr>
<tr>
<td>30ng/ml</td>
<td>42</td>
<td>36</td>
<td>287±31</td>
<td>0.47</td>
</tr>
<tr>
<td>300ng/ml</td>
<td>18</td>
<td>17</td>
<td>269±42</td>
<td>0.18</td>
</tr>
<tr>
<td>1000ng/ml</td>
<td>28</td>
<td>27</td>
<td>255±35</td>
<td></td>
</tr>
</tbody>
</table>

Table 10-2 Fibre growth and follicle longevity for follicles maintained Control Medium B or in medium supplemented with various concentrations of insulin-like growth factor-I.

Under the experimental conditions used in this work, IGF-I does not have a role in the regulation of wool production or follicle maintenance. This is in contrast with the reported effects of IGF-I on human hair growth in vitro, where significant stimulation of fibre growth rate was observed (Philpott et al. 1994).

The present findings also contrast with the following evidence. Murashita et al. (1993) have shown that skin keratinocytes possess IGF-I receptors and respond to IGF-I. This suggests a role for this growth factor in skin. In addition, IGF-I gene transcripts have been identified in anagen rat hair follicles (Little et al. 1994) and IGF-I receptors occur in the dermal papilla and germinal matrix of New Zealand Wiltshire wool follicles (Nixon et al. 1994a). The upregulation of IGF-I...
receptor number in response to a hormonal stimulus (Nixon et al. 1995a) provides additional support for this hypothesis. Nonetheless, no direct effects of IGF-I have been found on replicating cell numbers in the bulbs of wool follicles (Harris et al. 1993). There is some evidence that IGF-I may be involved in the transition of wool follicles from anagen to telogen (Nixon et al. 1995a), rather than in the regulation of fibre growth. Under these circumstances, no effects on the rate of fibre production would be observed using the in vitro follicle model, although this would be apparent as a reduction in the proportion of viable follicles. This was not apparent in the current study. Therefore a role for IGF-I in regulating wool growth or the follicle growth cycle is not supported by this study.

10.4 Fibroblast Growth Factor

The fibroblast growth factor (FGF) family is composed of a number of heparin-binding proteins which exist in a wide variety of tissues (Obana and Ito 1993) and promote cell proliferation and differentiation (du Cros et al. 1993). Basic FGF is mitogenic for keratinocytes in vitro (Norman et al. 1989) and both acidic and basic forms of FGF cause proliferation of dermal fibroblasts (du Cros et al. 1993). There is evidence that FGFs are expressed in the skin of sheep (Sutton et al. 1995) and that FGF receptors occur in the developing dermal papilla and hair bulb (Panaretto 1993). The presence of bFGF in the outer root sheath of the fibre follicle and in the basement membrane adjacent to the proliferative zone of the follicle bulb, may indicate specific growth-promoting functions for this growth factor. Both FGFs stimulated cell proliferation and differentiation in cultured follicle cells (Obana and Ito 1993).

However, the limited work with FGF in tissue culture (Fischer et al. 1980) has shown that 5-20ng/ml FGF had no measurable effects on epidermal growth or fibroblast contamination. Scandurro et al. (1993) found that maintenance of hair follicles lacking a dermal papilla in medium containing 10ng/ml bFGF, restored activation of procollagenase which may mediate dermal papilla-matrix cell interactions.

Given the potential for FGF to regulate the signalling of the follicle dermal papilla, the effects of bFGF on isolated wool follicles were examined in this chapter.

10.4.1 Experimental Procedure

Isolated wool follicles from a Drysdale sheep were maintained in Control Medium B or Control Medium B supplemented with 1, 10 or 100ng/ml fibroblast growth factor. The FGF used was defined as brain derived basic FGF, synthesised by Auspep Pty Ltd, Victoria, Australia (Cat.No. 2554) and consisted of amino acids 1-24. The lyophilised powder was reconstituted with Control Medium B.

10.4.2 Results and Discussion

Basic FGF inhibited the rate of wool production of isolated follicles by up to 50% (Table 10-3). There was no significant effect of this growth factor on follicle viability.
The data demonstrate for the first time that bFGF has direct inhibitory effects on the rate of wool production by isolated follicles. The presence of FGF receptors in the dermal papilla and hair bulb of mice (Panaretto 1993) support this finding.

In previous studies this growth factor has been shown to stimulate cell proliferation and differentiation in cultured follicle cells (Obana and Ito 1993) and dermal fibroblasts (dueros et al. 1993). In contrast, Imai et al. (1992) showed that bFGF had no effect on DNA synthesis in follicle bulb cells. It seems likely that bFGF may mediate dermal papilla-matrix cell interactions through the activation of collagenases, as it restores activation of procollagenase in co-cultures of dermal papilla with hair follicles lacking a dermal papilla (Scandurro et al. 1993). FGFs are expressed in the skin of sheep throughout the development of skin (Sutton et al. 1995) which supports a role for FGF in the ingress of developing follicular structures in foetal skin through the synthesis of proteases. The secretion of proteases by follicles maintained in tissue culture is likely to have disruptive effects on the follicle basement membrane and collagen sheath and, as a consequence, affect the growth of fibres in isolated follicles.

10.5  **Epidermal Growth Factor**

Epidermal growth factor (EGF) is another growth factor with the potential to affect wool production. Receptors for EGF have been detected in numerous tissues including keratinocytes (O'Keefe et al. 1982) and the epithelial components of hair and wool follicles (du Cros et al. 1992). EGF has been detected in the dermal papilla (Panaretto 1993), sebaceous glands and outer root sheath of fibre follicles, primarily in the zone of keratinisation (du Cros et al. 1992). In vivo, inhibition of wool production by EGF, with changes in keratin composition, a reduction in fibre strength, fleece shedding and changes in follicle morphology having been reported (Frenkel et al. 1975; Gillespie et al. 1982; Moore 1982; Behrendt et al. 1993).

EGF has also been reported to have slight mitogenic effects on follicles maintained in vitro (Rogers et al. 1987; Weinberg et al. 1990), although Philpott et al. (1991) reported no significant effects of EGF on hair follicle length after five days in culture. In contrast, other researchers (Hynd et al. 1994; Bond et al. 1994b) have found that maintenance of isolated wool
follicles in medium supplemented with 10-50ng/ml EGF has resulted in the cessation of fibre growth. EGF, at 25ng/ml, enhanced the release of collagenolytic activity from hair follicle cells, a response that is specific to fibre follicles (Weinberg et al. 1990). Nonetheless, EGF at 5-30ng/ml, is generally accepted to stimulate epidermal cell growth and prolong culture life in a dose-dependent fashion (Fischer et al. 1980; Thompson et al. 1985; Breidahl et al. 1989). In a review, Breidahl et al. (1989) stated that a distinct advantage was obtained with EGF, in the range of 10-100ng/ml, on stimulating keratinocyte cells. Enhanced culture life and reduced terminal differentiation were also noted.

In this section, the effects of supplementing the culture medium with 10-1000ng/ml EGF, on fibre growth and follicle viability of isolated wool follicles, were examined.

10.5.1 EXPERIMENTAL PROCEDURE
Wool follicles were isolated from one Drysdale sheep and maintained in Control Medium B or Control Medium B supplemented with 10, 100 or 1000ng/ml EGF (Sigma Chemical Co.). The rate of wool growth and the proportion of follicles which remained viable were determined.

10.5.2 RESULTS AND DISCUSSION
The presence of EGF in the tissue culture medium reduced growth rate by approximately 30% at all concentrations. No effects on follicle viability were observed (Table 10-4).

<table>
<thead>
<tr>
<th>EGF Concentration</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± s.e.m.) (µm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Medium B (no EGF)</td>
<td>23</td>
<td>18</td>
<td>251±36</td>
<td></td>
</tr>
<tr>
<td>10ng/ml EGF</td>
<td>24</td>
<td>20</td>
<td>171±20</td>
<td>0.09</td>
</tr>
<tr>
<td>100ng/ml EGF</td>
<td>24</td>
<td>22</td>
<td>168±17</td>
<td>0.05</td>
</tr>
<tr>
<td>1000ng/ml EGF</td>
<td>24</td>
<td>24</td>
<td>189±19</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Table 10-4 Fibre growth rate of follicles maintained in Control Medium B or in Control Medium B supplemented with various concentrations of epidermal growth factor.

The results are supported by the findings of Bond et al. (1994b) and Hynd et al. (1994), who showed that the addition of EGF to a wool follicle culture medium caused a reduction or cessation of fibre growth. This effect is supported by in vivo results which have shown that EGF inhibits wool production (Moore 1982; Hynd 1989b) with accompanying changes in keratin composition (Frenkel et al. 1975; Gillespie et al. 1982), reduced fibre strength (Behrendt et al. 1993) and altered follicle morphology (Gillespie et al. 1982; Moore 1982). It appears that many of these effects are mediated by EGF induced collagenase activity (Rogers et al. 1987).
10.6 Transforming Growth Factor alpha

The transforming growth factors (TGFs) are members of the epidermal growth factor (EGF) family of polypeptide growth factors. TGFα binds to the EGF receptor triggering the EGF pathway of receptor activity (Panaretto 1993; Wynn et al. 1989). Both the alpha and beta forms of TGF may stimulate or inhibit growth depending on the cell type and the presence and activity of other growth factors (Norman et al. 1989). TGFα is synthesised and secreted by keratinocytes. The addition of TGFα or EGF to keratinocytes in vitro induces TGFα gene expression suggesting an autocrine regulation of cell proliferation occurs (Norman et al. 1989; du Cros et al. 1992).

TGFα has been implicated in hair growth. In sheep follicles, TGFα was localised in the innermost cells of the outer root sheath and in cortical cells in the keratogenous zone, but was not found in inner root sheath or dermal papilla cells (Nixon et al. 1995b). In mice, TGFα has been shown to retard hair growth (Wynn et al. 1989) but in cultured follicles, cell proliferation has been stimulated by TGFα (Weinberg et al. 1990). Indeed, isolated follicles showed a dose-dependant increase in thymidine incorporation when the culture medium was supplemented with 10-50ng/ml TGFα (Weinberg et al. 1990).

In this section, the effect of maintaining isolated wool follicles in medium supplemented with 10-1000ng/ml TGFα will be examined.

10.6.1 Experimental Procedure

Isolated wool follicles from a Drysdale sheep were maintained in Control Medium B or Control Medium B supplemented with 10, 100 or 1000ng/ml transforming growth factor alpha. The TGFα was synthesised by Auspep Pty Ltd, Victoria, Australia (Cat.No. 1078) and consisted of rat TGFα, amino acids 34-43. The lyophilised powder was reconstituted with Control Medium B.

10.6.2 Results and Discussion

Maintenance of isolated wool follicles in nutrient medium supplemented with TGFα reduced the rate of fibre growth by approximately 20%. This trend was not statistically significant (Table 10-5). The viability of isolated follicles was not affected by the presence of TGFα.
Table 10-5 Fibre growth rate by follicles maintained in Control Medium B or in Control Medium B supplemented with various concentrations of transforming growth factor alpha.

<table>
<thead>
<tr>
<th>TGF-α Concentration</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (μm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Medium B (no TGFα)</td>
<td>21</td>
<td>20</td>
<td>388±43</td>
<td></td>
</tr>
<tr>
<td>10ng/ml TGFα</td>
<td>21</td>
<td>20</td>
<td>309±41</td>
<td>0.16</td>
</tr>
<tr>
<td>100ng/ml TGFα</td>
<td>20</td>
<td>20</td>
<td>313±51</td>
<td>0.28</td>
</tr>
<tr>
<td>1000ng/ml TGFα</td>
<td>21</td>
<td>20</td>
<td>334±41</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Whilst there was no significant reduction of fibre growth in the presence of TGFα, the trend is towards a reduction in growth. Inhibition of fibre growth in sheep follicles concurs with the findings of Wynn et al. (1989) who showed that TGFα inhibits hair growth in mice. Nixon et al. (1995b) have recently identified TGFα in wool follicles. Together, these observations provide some support for the hypothesis that TGFα is involved in the regulation of wool follicles and that it may have some influence on the control of fibre production.

The mechanism of action of TGFα may involve activation of the EGF biochemical pathways. Binding of TGFα to cell membrane EGF receptors has been previously described (Wynn et al. 1989; Panaretto 1993). The effects of EGF-type mechanisms, on protease activation, are likely to affect fibre growth in vitro through damage to the collagen sheath and basement membrane.

10.7 ETHANOLAMINE / PHOSPHOETHANOLAMINE

The effects on wool growth of supplementing the follicle culture medium with the pituitary growth factors, phosphoethanolamine and ethanolamine, were examined in this section. Most pituitary growth factors act by stimulating the secretion of other growth factors. These pituitary factors may not, therefore, have direct effects on follicles and, consequently, they may be ineffectual in a follicle culture system (Wallace 1979). In spite of this, effects of phosphoethanolamine and ethanolamine on cultured follicles were examined because of their reported mitogenic activity. For example, these growth factors have been shown to be potent keratinocyte mitogens (Hawley-Nelson et al. 1980; Breidahl et al. 1989), with ethanolamine having a higher growth promoting activity than phosphoethanolamine (Breidahl et al. 1989).

10.7.1 EXPERIMENTAL PROCEDURE

Isolated follicles from one Romney sheep were maintained in Control Medium B or Control Medium B supplemented with a 50:50 mixture of phosphoethanolamine and ethanolamine (10⁻⁵M). The effects of this supplement on fibre growth and follicle viability were determined.
10.7.2 RESULTS AND DISCUSSION

Supplementation of the culture medium with ethanolamine and phosphoethanolamine increased the rate of fibre production by approximately 40%. However, this was not statistically significant. These growth factors had no effect on follicle viability (Table 10-6).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± s.e.m.) (μm/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Medium B</td>
<td>17</td>
<td>14</td>
<td>144±29</td>
<td></td>
</tr>
<tr>
<td>plus pituitary components</td>
<td>18</td>
<td>16</td>
<td>206±29</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table 10-6 Growth of isolated wool follicles maintained in Control Medium B or Control Medium B supplemented with a mixture of ethanolamine and phosphoethanolamine

The data suggest that the pituitary factors may have a direct regulatory effect on wool follicles. There is no in vivo information on the effect of these growth factors on wool growth. However, removal of the pituitary gland results in the cessation of wool production (Wallace 1979), whilst administration of a pituitary extract, free of thyrotropic activity, stimulates fibre growth (Ferguson et al. 1965). The data in this section suggest that ethanolamine and phosphoethanolamine may have some potential in stimulating wool production and that they should be considered as potential wool growth regulators. Indeed, further in vivo and in vitro work to establish whether these factors are important wool growth regulators would be beneficial.

10.8 MINOXIDIL

Minoxidil (6-amino-1, 2-dihydro-1-hydroxy-2-imino-4-piperidino-pyrimidine) acts pharmacologically as both a vasodilator and a growth stimulant for human hair (Baden et al. 1988; Price and Menefee 1990; Dooley et al. 1991). Details of the mechanism of action of this compound are currently not available. Initial reports speculated that vasodilation underlies both actions and that minoxidil affects hair growth by increasing blood flow to the hair follicle. Later studies have suggested a direct action of minoxidil on the follicle itself. A number of studies have also shown that sulphation of minoxidil must occur to activate the growth factor (Buhl et al. 1989; Imai et al. 1992) and that minoxidil sulphotransferase is located within the cytoplasm of cells in the lower outer root sheath of fibre follicles (Dooley et al. 1991; Baker et al. 1991; Waldon et al. 1993). This site appears to be the target of minoxidil action.

Supplementation of tissue culture media with minoxidil has identified a direct effect of this compound on fibre follicles. Rat vibrissae follicles maintained in minoxidil supplemented media increased in length six times faster than control follicles (Buhl et al. 1989). Human hair follicles maintained in vitro grew faster in 200ng/ml minoxidil (Philpott et al. 1990). However minoxidil has also been shown to be ineffective at 10μg/ml and significantly inhibited hair growth at 200μg/ml. Nevertheless, the longevity of follicles in the presence of minoxidil was greater than control follicles (Philpott et al. 1990). Minoxidil supplementation at 50 - 200μg/ml was also
found to increase cysteine and glycine incorporation into the follicle whereas higher doses were inhibitory (Buhl et al. 1989). Minoxidil also induced proliferation of hair epithelial cells near the base of the follicle (Buhl et al. 1987) and increased DNA synthesis of hair bulb cells in vitro (Imai et al. 1992). All these findings indicate there is a direct effect of minoxidil on hair follicles and suggest that the mechanism of action is independent of blood flow.

Recent in vivo rat studies which incorporate the topical application of minoxidil suggest that minoxidil may not increase the rate of hair growth, but rather increases the duration of anagen by shortening the time that the hair follicle is in the resting growth cycle stage (Frienkel et al. 1989). The action of minoxidil on human hair is that it keeps trichocytes of the hair bulb in anagen longer, which consequently allows hair fibres to attain a greater length (Baden et al. 1988). This is supported by studies in sheep, where administration of minoxidil reduced wool growth rate, fibre length, fibre diameter and rate of bulb cell division (Hynd and Ehrhardt 1987).

All these observations suggest that the minoxidil effects may be via changes in the hair cycle. This section examines whether 200ng/ml, 10μg/ml and 200μg/ml minoxidil affect wool fibre growth and follicle viability.

10.8.1 EXPERIMENTAL PROCEDURE

Wool follicles were isolated from one Drysdale and one English Leicester sheep. Approximately 24 follicles from each animal were maintained in Control Medium B or Control Medium B supplemented with 200ng/ml, 10μg/ml or 200μg/ml minoxidil.

10.8.2 RESULTS AND DISCUSSION

The inclusion of 200μg/ml minoxidil in the tissue culture medium had a significant inhibitory effect on the rate of fibre production by both English Leicester and Drysdale wool follicles (Table 10-7). In both instances the rate of fibre growth was half that produced by follicles maintained in minoxidil-free medium. This concentration of minoxidil also significantly reduced the viability of follicles from the English Leicester but not the Drysdale sheep.

In follicles isolated from the Drysdale sheep, minoxidil supplementation at 10μg/ml and 200ng/ml had no effect on fibre growth. The supplementation of nutrient medium with 10μg/ml also had no effect on wool growth by English Leicester follicles. However, there was a 30% reduction in the rate of fibre growth by these follicles in the presence of 200ng/ml minoxidil. This effect was not statistically significant.
EFFECT OF VARIOUS GROWTH FACTORS

Table 10-7 Fibre growth rate by follicles maintained in Control Medium B or in Control Medium B supplemented with various concentrations of minoxidil. Differences in the proportion of viable follicles were determined using $\chi^2$ analysis; ** $P<0.01$.

<table>
<thead>
<tr>
<th></th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± S.E.M.) ($\mu$m/day)</th>
<th>P value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drysdale follicles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Medium B (no minoxidil)</td>
<td>24</td>
<td>23</td>
<td>368±36</td>
<td></td>
</tr>
<tr>
<td>200ng/ml minoxidil</td>
<td>24</td>
<td>24</td>
<td>367±35</td>
<td>0.98</td>
</tr>
<tr>
<td>10$\mu$g/ml minoxidil</td>
<td>24</td>
<td>24</td>
<td>395±37</td>
<td>0.62</td>
</tr>
<tr>
<td>200$\mu$g/ml minoxidil</td>
<td>24</td>
<td>24</td>
<td>202±16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>English Leicester follicles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Medium B (no minoxidil)</td>
<td>24</td>
<td>22</td>
<td>201±30</td>
<td></td>
</tr>
<tr>
<td>200ng/ml minoxidil</td>
<td>24</td>
<td>22</td>
<td>142±21</td>
<td>0.11</td>
</tr>
<tr>
<td>10$\mu$g/ml minoxidil</td>
<td>20</td>
<td>18</td>
<td>211±38</td>
<td>0.83</td>
</tr>
<tr>
<td>200$\mu$g/ml minoxidil</td>
<td>23</td>
<td>13</td>
<td>98±20</td>
<td>0.003</td>
</tr>
</tbody>
</table>

The results show that 200$\mu$g/ml minoxidil inhibits wool fibre production. Philpott et al. (1990) also found that the maintenance of human hair follicles in media supplemented with 200$\mu$g/ml minoxidil significantly restricted fibre production. This inhibition may be due to toxic effects of minoxidil at the high concentration rather than any physiological regulation. Follicle viability was also significantly reduced when English Leicester follicles were maintained in a culture medium supplemented with 200$\mu$g/ml minoxidil.

Philpott et al. (1990) identified that 10$\mu$g/ml minoxidil had no effect on fibre production in vitro, but in their hands 200ng/ml minoxidil increased the rate of hair growth by isolated scalp hair follicles; a finding not supported by this study. It may be that there are species specific differences in the effects of minoxidil. Administration of minoxidil to sheep in vivo reduced wool growth, fibre length, fibre diameter and the rate of bulb cell division (Hynd and Ehrhardt 1987). This may have been a result of the toxic minoxidil effects proposed above, or may signify a physiological effect of this growth factor in sheep.

Recent in vivo evidence suggests that the reported mitogenic effects of minoxidil are mediated through regulation of the follicle cycle, rather than the rate of fibre production. Minoxidil appears to maintain fibres in anagen resulting in fibres attaining a greater length, rather than affecting the rate of fibre growth (Baden et al. 1988; Frienkel et al. 1989). Consequently, if minoxidil regulation is via follicle cycle control, increases in the rate of fibre production in vitro would not be expected. Therefore, the data do not rule out the possibility that at specific doses, minoxidil may regulate wool growth via control of the follicle growth cycle. This study supports the hypothesis that the effects of minoxidil are not achieved through regulation of the rate of fibre growth.
It is also interesting to observe the very high viability rate for follicles, particularly those of Drysdale sheep, maintained with minoxidil. This may be due to minoxidil causing follicles to remain in an active growth phase when they may otherwise have regressed to telogen. Further work on this observation may lead to a greater understanding of the mechanisms by which wool growth is regulated.

10.9 Antler Extract

Deer antlers are organs synthesised on the frontal bones of the skull of male Cervidae. Each year the antlers grow, calcify, die, shed and then regenerate. Annual regeneration involves the rapid production of a highly proliferative tissue. During times of synthesis, tissue grows from the antler tip at the rate of 1-2cm per day and is almost immediately covered in a soft skin which supports a dense mat of fine hair (Sadighi et al. 1994). The synthesised tissue is highly vascularised and innervated. Work by Suttie et al. (1994) has shown that the antler tip is the most proliferative zone and that unidentified but potent stimulators of cell division are present in the aqueous extracts of this tissue. Suttie et al. (1994) examined the mitogenicity of extracts of antler tissue on antler cells in vitro and identified a peak of activity at 1.25 - 2.5mg/ml after which mitogenicity declined.

The rapid rate at which antler tissue is synthesised and the observation that hair follicles are initiated and fibre produced on the antler immediately following synthesis of this tissue, gave rise to the hypothesis that potent stimulators of fibre growth are likely to be present in the tip of regenerating deer antler. In this section, the mitogenicity of aqueous deer antler extract on isolated wool follicles was examined.

10.9.1 Experimental Procedure

Freeze dried aqueous extract of velvet antlers from New Zealand Red Deer (Cervus elaphus) was provided by Dr J.M. Suttie (AgResearch, Invermay Agricultural Centre, Mosgiel, New Zealand). Isolated wool follicles were maintained in Control Medium B or in Control Medium B aseptically supplemented with antler extract to a final concentration of between 0.2- 5mg/ml. In order to examine this complete concentration range in detail, three separate experiments were undertaken to examine portions of this range of antler extract concentrations. The three experiments were undertaken on consecutive days. On each occasion, media supplementation with antler extract was undertaken using the same 5mg/ml stock solution of extract prepared in Control Medium B.

10.9.2 Results and Discussion

The effect of low concentrations of antler extract on the rate of wool fibre growth in vitro was examined in Experiment One. There was a concentration dependent inhibition of wool growth with increasing antler extract supplementation up to 0.6mg/ml. However this was not statistically significant at any of the concentrations examined here (Table 10-S). In Experiment Two there was also concentration dependent inhibition of wool growth up to 2.0mg/ml of antler extract.
with the inhibition reaching statistical significance at the high end of the range ($P<0.05$). Data from Experiment Three showed that there was significant inhibition of fibre growth \textit{in vitro} by isolated wool follicles in the presence of 3.5-5mg/ml aqueous antler extract ($P<0.05$).

<table>
<thead>
<tr>
<th>Antler extract concentration</th>
<th>number of follicles isolated</th>
<th>number of follicles viable</th>
<th>fibre growth rate (mean ± s.e.m.) (µm/day)</th>
<th>$P$ value (growth rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment one</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Medium B (0mg/ml)</td>
<td>18</td>
<td>18</td>
<td>232±35</td>
<td>0.42</td>
</tr>
<tr>
<td>0.2mg/ml</td>
<td>22</td>
<td>19</td>
<td>196±27</td>
<td>0.30</td>
</tr>
<tr>
<td>0.4mg/ml</td>
<td>22</td>
<td>21</td>
<td>187±26</td>
<td>0.08</td>
</tr>
<tr>
<td>0.6mg/ml</td>
<td>25</td>
<td>24</td>
<td>158±27</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment two</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Medium B (0mg/ml)</td>
<td>21</td>
<td>17</td>
<td>229±35</td>
<td>0.76</td>
</tr>
<tr>
<td>0.5mg/ml</td>
<td>21</td>
<td>19</td>
<td>243±35</td>
<td>0.58</td>
</tr>
<tr>
<td>1.0mg/ml</td>
<td>22</td>
<td>20</td>
<td>206±29</td>
<td>0.16</td>
</tr>
<tr>
<td>1.5mg/ml</td>
<td>24</td>
<td>23</td>
<td>172±24</td>
<td></td>
</tr>
<tr>
<td>2.0mg/ml</td>
<td>22</td>
<td>20</td>
<td>141±15</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Experiment three</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Medium B (0mg/ml)</td>
<td>20</td>
<td>20</td>
<td>235±35</td>
<td>0.12</td>
</tr>
<tr>
<td>2.5mg/ml</td>
<td>23</td>
<td>21</td>
<td>187±20</td>
<td>0.08</td>
</tr>
<tr>
<td>3.0mg/ml</td>
<td>22</td>
<td>20</td>
<td>181±16</td>
<td>0.01</td>
</tr>
<tr>
<td>3.5mg/ml</td>
<td>22</td>
<td>20</td>
<td>158±15</td>
<td></td>
</tr>
<tr>
<td>4.0mg/ml</td>
<td>20</td>
<td>19</td>
<td>150±18</td>
<td>0.007</td>
</tr>
<tr>
<td>5.0mg/ml</td>
<td>18</td>
<td>18</td>
<td>132±17</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 10-8 Wool growth by isolated follicles maintained in Control Medium B or Control Medium B supplemented with aqueous antler extract. Data presented from three separate experiments examining the range 0-5mg/ml extract.

The rate of wool growth by isolated follicles maintained in Control Medium B (0mg/ml antler extract) was similar in each of the three experiments (Table 10-8), enabling fibre growth to be compared in each experiment. The growth rate values for follicles maintained in Control Medium B alone (0mg/ml extract) were averaged to enable the direct comparison of the wool growth rates presented in Figure 10-1. Data from each experiment are presented in a different colour.
Effect of Various Growth Factors

Figure 10-1  The rate of wool growth by isolated follicles maintained in Control Medium B (0mg/ml) or Control Medium B supplemented with different concentrations of antler extract. Data from Experiments 1-3 (Table 10-8). □ 0mg/ml antler extract data averaged for the three experiments; □ data from Experiment One; □ data from Experiment Two; □ data from Experiment Three. Statistical differences are reported relative to 0mg/ml antler extract (* P<0.05).

The graphical presentation of data in Figure 10-1 shows there is a concentration dependent inhibition of wool growth by the antler extract. There is also an inexplicable reduction in the rate of wool growth within each experiment which appears incongruous to the growth rate responses occurring between experiments. The higher extract concentration in each experiment was the most inhibitory on wool growth irrespective of when this occurred in the range of extracts examined. There is no obvious reason for the differences in the effect of the extract in the different experiments; all follicles were randomly assigned to extract concentrations, experiments were performed on consecutive days and a stock solution of extract was diluted for use in each experiment.

The data in this section show that aqueous antler extract had no stimulatory effect on wool growth from isolated follicles.

10.10 Discussion and Conclusions

Insulin had no effect on the rate of fibre growth by isolated wool follicles over three days. This concurs with the in vivo findings of Bray et al. (1990), Wynn et al. (1988) and Johnsson et al. (1985) that changes in wool growth are not necessarily correlated with, or dependent upon, changes in plasma insulin concentrations. In the in vivo environment, insulin may well have some role in the mediation of environmental effects on wool production through its effects on energy.
partitioning and protein synthesis. The study presented here, however, demonstrates that insulin does not have direct effects on the regulation of fibre production at the wool follicle.

Insulin-like growth factor-I (IGF-I) also had no effect on wool production in vitro which contrasts with the stimulatory effects documented for human hair growth in culture (Philpott et al. 1994). Cell receptors for IGF-I have been identified in wool follicles (Nixon et al. 1994a) and these have been shown to upregulate in response to a hormonal challenge (Nixon et al. 1995a). However this is typically prior to transition of the follicle from anagen to telogen rather than changes in the rate of fibre production (Dicks and Williams 1993; Nixon et al. 1995a). In wool follicles it therefore appears that IGF-I is not involved in the regulation of fibre growth rate, but may have a role in regulation of the follicle growth cycle. This deserves further investigation.

Fibroblast growth factor (FGF), epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) each inhibited fibre growth in vitro, probably by an identical mechanism. Inhibition of fibre growth by 45% (P=0.003) occurred when the nutrient medium was supplemented with 10ng/ml FGF. Other concentrations of FGF also caused significant wool growth inhibition. Fibre growth was reduced by 33% (P=0.05) in the presence of 100ng/ml EGF with a trend towards an inhibition of wool growth at other concentrations. A lesser effect was observed when the nutrient medium was supplemented with TGFα. Nevertheless, fibre growth was reduced 20% in the presence of 10ng/ml (P=0.16) with no effect being detected at other concentrations. These findings are supported by other results which indicate that fibre production by isolated wool follicles and murine follicles are inhibited by EGF (Bond et al. 1994b; Hynd et al. 1994) and TGFα (Wynn et al. 1989).

The effect of these growth factors on wool growth appears to be through the activation and synthesis of dermal collagenases. Fibroblast growth factor (Scandurro et al. 1993) and EGF (Rogers et al. 1987) have each been shown to enhance follicle collagenase activity. TGFα binds to the EGF receptor and by this mechanism may also activate collagenase synthesis (Panaretto 1993; Wynn et al. 1989). Collagenase probably has a role in the ingress of follicles into the dermis during foetal development (Sutton et al. 1995) and may also regulate ingress during reactivation of telogen follicles. The inhibition of wool growth rate noted in isolated follicles may be due to the disruption of collagen structures, particularly in the follicle collagen sheath. Examination of the role of these growth factors in regulating follicle activation and cycling should provide valuable information on the mechanisms regulating these events.

The pituitary components ethanolamine and phosphoethanolamine at 10⁻⁵M stimulated fibre production in isolated follicles by about 40% (P=0.16). Further experimentation is required to validate this observation given that removal of the pituitary prevents wool production and administration of a pituitary extract stimulates wool production (Ferguson et al. 1965; Wallace 1979). The mitogenic effects of these factors are certainly worth further investigation.

Supplementation of the nutrient medium with 200µg/ml minoxidil significantly inhibited wool growth by follicles isolated from both English Leicester (P=0.003) and Drysdale (P<0.001) sheep. There was no significant effect of minoxidil on wool production at any other
concentration examined. This concentration of minoxidil was also found to inhibit fibre production by human hair follicles (Philpott et al. 1990). In human hair follicles maintained in vitro, 200ng/ml minoxidil stimulated hair production. However this concentration was found to have no effect on the rate of fibre growth by isolated wool follicles. It is hypothesised that this difference is a species specific effect. However there is some evidence which suggests that minoxidil acts by regulating the hair growth cycle rather than fibre growth. Minoxidil has been shown to reduce the duration of the telogen phase of follicles (Frienkel et al. 1989) and to maintain follicle bulb trichocytes in the anagen phase for an increased length of time (Baden et al. 1988). These results suggest that fibre growth is regulated by direct effects on the follicle via changes in the activity of the hair follicle rather than increased fibre production. The inhibition of fibre growth noted in this work and by Philpott et al. (1990), may be due to the toxic effects of this growth factor at high concentrations.

Aqueous extracts of deer antler had no stimulatory effects on fibre growth in cultured wool follicles. Indeed, high concentrations of this extract (3.5-5ng/ml) were inhibitory for fibre production. There are a number of possible reasons for this lack of wool growth stimulation. It may be due to between species differences in the conformation of potential mitogens (Lehnlinger 1975). Alternatively, immunological interference by other water soluble components from the deer may be obscuring any mitogenic effects on the wool follicles. Properdin, conglutinin and acute phase proteins are all serum proteins which provide immunological protection but may interfere with cell growth in a tissue culture environment (Humphrey and White 1970). Complement may also affect cell growth in vitro (Humphrey and White 1970). It would be worth investigating the effect of heat inactivating the antler extract, on in vitro wool growth.

In conclusion, a number of growth factors affect the rate of wool growth in cultured follicles. Fibroblast growth factor, epidermal growth factor and transforming growth factor alpha inhibit fibre production. The most likely mechanism of action is the disruption of follicle morphology due to collagenases which are activated by the growth factors themselves. Minoxidil inhibits fibre growth at high concentrations which may reflect a toxic effect of this growth factor. Alternatively, minoxidil may inhibit fibre growth via the outer root sheath, the target site for minoxidil. The only stimulation of fibre production in this study was through supplementation with the pituitary components, ethanolamine and phosphoethanolamine. Potential mechanisms of action of these factors requires further investigation. This study has also shown that a number of growth factors achieve their effects by regulating wool production through direct actions on the wool follicle growth cycle. However no factor has been identified which regulates the growth rate of the fibre itself.
The aim of science is to seek the simplest explanations of complex facts. We are apt to fall into the error of thinking that the facts are simple because simplicity is the goal of our quest. The guiding motto in the life of every natural philosopher should be, "Seek simplicity and distrust it."

Alfred North Whitehead
11 GENERAL DISCUSSION

11.1 Evaluation of the Follicle Culture Technique 151

11.2 Significant Outcomes of this Study 153

11.3 Potential for Future Research 158
11 GENERAL DISCUSSION

11.1 EVALUATION OF THE FOLLICLE CULTURE TECHNIQUE

The wool follicle culture system developed in this thesis details conditions whereby isolated follicles were successfully maintained \textit{in vitro}, with the continuous production of a typical wool fibre for at least three days. Fibre growth was significantly reduced when follicles were maintained in buffered saline. This shows that the growth of wool in culture requires the provision of nutrients and responds to culture conditions, rather than being the continuation of some process already underway in the follicle. This is essential for the \textit{in vitro} system to be considered a legitimate model of \textit{in vivo} fibre growth.

The structure of wool fibres produced in culture was typical of those grown \textit{in vivo}. Using scanning electron microscopy, it was apparent that the distance between consecutive cuticle ridges of freshly isolated fibres and of fibres produced during maintenance of follicles in tissue culture, were the same. This demonstrates that the external physical structure of fibres is retained in culture and that the formation of a normal wool fibre occurs. Examination of follicles and fibres using a light microscope concurred with this observation. In addition, bromodeoxyuridine incorporation showed that cells in the germinative matrix of the follicle bulb remained mitotically active during \textit{in vitro} maintenance.

Amplification of keratin DNA was undertaken by \textit{in situ} PCR. This is the first report of the use of this technique in wool follicles. \textit{In situ} PCR keratin gene amplification showed that structurally intact DNA was present within all cells of the wool follicle after three days maintenance \textit{in vitro}. From all of the evidence, I am confident that the maintenance of wool follicles in culture for three days does not disrupt the production of a normal fibre.

Techniques for isolating wool follicles were evaluated in the current study. I believe microdissection to be the most appropriate method for isolating wool follicles. While it is reasonably time-intensive, it is superior to plucking, shear isolation and collagenase tissue digestion. Microdissection enables follicles to be collected which have not been damaged and, consequently, a high proportion continue to produce a wool fibre in culture. In contrast, the structure of a follicle is damaged when obtained by plucking (Weterings \textit{et al.} 1982; Green \textit{et al.} 1986), shear isolation (Philpott \textit{et al.} 1989) or protease digestion (Rogers \textit{et al.} 1987).

I believe that the optimum technique for measuring the rate of wool growth by isolated follicles is that of fixed time analysis. This was superior to analysis by regression because, while the average growth rate was identical using either technique, fixed time analysis was less time intensive and less disruptive to the culture conditions. That is, the use of fixed time analysis halved the number of times culture plates were removed from the incubator. I therefore recommend fixed time analysis for the measurement of fibre growth in culture.

The composition of the medium in which isolated follicles were maintained affected the rate of wool growth and the viability of follicles. I believe that the optimum conditions for growing wool...
in vitro can be defined as William’s Medium E supplemented with insulin, hydrocortisone, sodium selenite and transferrin. Supplementing with trace elements, Fungizone®, penicillin or streptomycin reduced in vitro wool growth. Foetal calf serum had no effect on in vitro fibre growth under the conditions in the current study; an observation which concurs with the findings of Boyce and Ham (1983).

During the course of this study I was not able to identify why follicles ceased to produce fibres after one week in culture. It may be that, in response to the culture conditions, follicles undergo a transition to the telogen phase of the growth cycle, or that isolation from the dermis results in a lack of autocrine and paracrine messages. This may then cause trichocytes to lose their specialisation and revert back to the dermal/epidermal tissue components from which they were initiated. In a similar manner, the recognition and association of cells of the same type is crucial to the maintenance of tissue integrity in vivo (Edelman and Thiery 1985). This cessation of fibre growth does not appear to be due to the depletion of nutrients in the culture medium, as daily renewal of the medium (preliminary data not presented here) does not increase follicle longevity.

A further observation to be addressed is the wide range of wool growth rates. It may be that some follicles were slightly damaged during isolation, although this was not apparent under microscopic examination. In addition, bromodeoxyuridine uptake studies showed that both fast- and slow-growing follicles had cellular patterns of DNA synthesis identical to those observed in vivo. This suggests that fibre growth in cultured follicles originate from normal cell division in the follicle bulb, even in slow growing follicles. From this evidence, I believe that the events responsible for the wide range of fibre growth rates have no potential to compromise the normal process of fibre production and trichocyte proliferation. Indeed, it may be that the range of fibre growth rates noted in vitro are merely a continuation of events occurring in vivo. The range of individual fibre length growth rates within any one animal is 260-694μm/day for Romney sheep (Woods pers.comm.). This may account for some growth rate variability.

An alternate mechanism which may be involved in the in vitro growth rate variability is that at the time of isolation, individual follicles may be under the regulation of specific growth factors and that the culture conditions may not be suitable for the continued production of these factors. If this were the case, follicles with a greater stored concentration of an undefined growth factor may continue to produce fibre for a longer period, and potentially at a greater rate, than follicles with a lower concentration of the stored growth factor at the time of isolation. Nonetheless, wool growth by isolated follicles, maintained in vitro, occurred at a consistent rate for at least three days, with the rate of fibre production being approximately half of that occurring in vivo.

The ratio of in vivo:in vitro fibre growth was consistent over the different sheep genotypes examined in this study. This is the first report of a between breed comparison of in vitro fibre growth. It was apparent that follicle curvature, small follicle size and a high density of follicles in the skin, each increased the difficulty of isolation and reduced in vitro follicle viability. From these results, I would recommend the use of coarse-wool breeds of sheep for future in vitro studies of wool growth.
In addition to the greater ease of follicle isolation from coarse-wool breeds, the rate of fibre growth is also likely to be enhanced. In this study I found that for isolated follicles there was a significant, positive correlation between the \textit{in vitro} fibre growth rate and follicle bulb diameter. This concurs with numerous reports of an \textit{in vivo} relationship between follicle bulb size and fibre growth rate (for example Henderson 1965; Black and Reis 1979; Kelly \textit{et al}. 1993). The relationship between the \textit{in vitro} rate of wool growth and follicle size was best explained using a split trendline. This provides some support for the idea of differential control of primary and secondary follicles. Differences in the fibre growth cycles have been reported for the two follicle populations (Hardy 1992). Therefore it is feasible that different regulatory mechanisms occur. This could be examined, in future work, by distinguishing primary and secondary follicles at isolation on the basis of size or associated accessory structures. For example, secondary follicles have no sweat glands or arrector pili muscle (Chapman and Ward 1979).

In conclusion, I believe that this study demonstrates that wool follicles can be successfully isolated from sheep and, given appropriate culture conditions, will continue to grow wool at a linear rate for at least three days. The fibre produced has a structure which is typical of wool produced under normal \textit{in vivo} conditions. I believe that the \textit{in vitro} wool growth model described here provides an appropriate, effective method of evaluating mechanisms with the potential to regulate wool growth \textit{in vivo}.

11.2 \textbf{SIGNIFICANT OUTCOMES OF THIS STUDY}

I believe that the key finding of this study is that the regulation of wool growth occurs through responses to circulating extra-follicular factors, rather than modifications to systems within the wool follicle. The isolation of wool follicles and their \textit{in vitro} maintenance under conditions which are conducive to continued wool growth, resulted in the release of follicles from the systemic influences controlling fibre growth. Loss of this regulation was demonstrated following both seasonal and nutritional manipulation. In addition, the provision of extra-follicular factors by the inclusion of ovine serum influenced follicle wool production in a season dependent manner.

\textit{In vivo}, wool production is influenced by season, with most sheep breeds having a greater wool growth rate during summer than winter (Coop 1953; Bigham \textit{et al}. 1978; Geenty \textit{et al}. 1984; Lincoln 1990). \textit{In vivo} wool growth by the four English Leicester sheep used in this study concurred with these observations. Peak wool production occurred during summer (1.53mg/cm\(^2\)/day) with a nadir during winter (0.64mg/cm\(^2\)/day). Isolation of wool follicles from these sheep at regular intervals throughout the year, showed that the seasonal regulation of fibre growth was disrupted, when follicles were removed from the extra-follicular influences within the animal and maintained \textit{in vitro}. Isolated follicles grew at a rate which was independent of the time of year.

Similar losses of systemic regulation were observed when animals were maintained on different nutritional treatments. Animals consuming high energy, high protein diets produced 36\% more wool on a clipped patch, than those on a control diet (1.48 vs 1.09mg/cm\(^2\)/day; \(P=0.04\)).
contrast, when follicles were isolated from these animals and maintained in vitro, wool growth rates were identical (P=0.95). This data shows that under both the seasonal and nutritional conditions described here, the in vitro wool growth rate was independent of any of the environmental influences which affected the animal at the time of follicle isolation.

Further evidence for the suggestion that environmental regulation occurs via extra-follicular factors is that the rate of wool growth by isolated follicles was inhibited by ovine serum. This inhibition was season dependent, with serum collected during autumn having a more inhibitory effect on fibre growth than serum obtained during spring. I suggest that this finding implies that the environmental regulation of wool growth is achieved through direct effects of extra-follicular factors on follicle growth processes. It may be that endocrine mechanisms exist in winter to suppress wool growth and, as a consequence, to conserve the limited amount of available nutrients for more vital processes necessary for survival. At other times of the year, when nutrients are more readily available, a change in the nutrient partitioning serum 'factor' would allow increased wool growth. It would be interesting to undertake a multi-factorial analysis of growth responses for follicles isolated at different times of the year and supplemented with serum from different seasons.

An alternate mechanism to that of a nutrient partitioning 'factor' is endocrine control of vasodilation and blood flow, and by this mechanism, dermal temperature. That is, ovine serum may contain factors capable of increasing or decreasing vasodilation in a season-dependent manner. While the in vitro data presented in this study show that a reduced temperature significantly reduces in vitro fibre growth (P<0.001), this mechanism cannot be responsible for the reduction in fibre growth resulting from ovine serum supplementation, in the tissue culture environment. However, this does not rule out the potential of temperature and/or blood flow as a mechanism for the in vivo regulation of wool growth. For example, exposure of sheep to a cold environment has resulted in increased (Hopkins and Richards 1979), reduced (Coop 1953; Bennett et al. 1962; Wallace 1979; Hopkins and Richards 1979) or unaltered (Bottomley 1979) rates of wool production. Increases in wool growth under these conditions have been correlated with increased feed intake (Bottomley 1979). This evidence suggests that wool growth regulation occurs through a nutrient partitioning mechanism.

The findings of this study support the diminishing returns scenario of Scobie and Woods (1992) regarding formation of a fibre medulla. Scobie and Woods (1992) suggest that a medulla results when the rate of production of fibre cells exceeds their ability to synthesise keratin proteins. That is, as fibre volume increases, there is insufficient keratin to form any type of cortical cell and a medulla forms. Conversely, as fibre volume decreases, the occurrence of a medulla is reduced. In the current study, the in vitro growth of wool follicles resulted in a reduction of both wool fibre volume and medulla volume. This supports the hypothesis of Scobie and Woods (1992). Certainly, the observation that growing wool follicles in vitro affects the degree of fibre medullation, may provide a model suitable for examining potential factors responsible for the initiation and maintenance of a medulla in vivo.
A further interesting observation in this study was that during winter there was a reduction in the proportion of follicles which remained viable in culture. This led to the suggestion that a higher proportion of the follicles which were isolated during winter were in a catagen or telogen phase of the follicle growth cycle. A formula was therefore developed to determine whether the reduction in the in vivo wool growth rate during winter was completely accounted for by changes in fibre diameter and volume. The data indicate that the winter reduction in in vivo wool production was not fully accounted for by a decrease in fibre size. Therefore, it seems likely that a reduction in the proportion of follicles in anagen occurs during winter. This is supported by Parry et al (1991) who showed that in New Zealand Wiltshire sheep, the proportion of follicles in anagen was reduced during winter. Consequently, the reduced proportion of follicles in anagen may be reflected in the reduction of in vitro follicle viability, during winter. The growth of wool follicles in culture therefore provides a model of wool growth in which factors controlling the follicle growth cycle may be investigated.

While it cannot be ruled out that some cellular modification of the follicle is occurring in a season dependent manner, I believe that there is a considerable body of evidence to support the hypothesis that wool growth regulation is effected via endocrine mechanisms. Given that ovine serum had an inhibitory effect on fibre growth it appears that wool growth regulation occurs via follicle inhibition rather than stimulation. An attempt was made in this study to define the active components in ovine serum.

Ovine serum which had been collected during winter was fractionated into its component proteins on the basis of molecular size and charge. From this study it was apparent that specific fractions, and consequently specific protein or protein-bound molecules, confer the observed inhibition of wool fibre growth. These were tentatively identified as a 350kD protein (possibly an α₁-lipoprotein) or a 130kD protein which could not be identified. A further component which conferred significant wool growth inhibition was a 55kD protein which has tentatively been identified as albumin on the basis of size and affinity binding. No attempt was made to purify these protein fractions. Further work is needed to identify the specific active components of these serum fractions. It is possible that one of the less dominant protein components in the unpurified fractions was responsible for the observed inhibition. Likely candidates would be the endocrine growth factors. However their molecular size (approximately 3 to 21kD) suggests that, if the growth factors are responsible for the regulation of wool growth, they are transported while bound to other protein components in serum.

The identity and mode of action of some potential candidates which may contribute to the endocrine regulation of wool growth were investigated. Basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) each inhibited wool fibre growth in vitro.

The potential mechanism by which bFGF affects fibre growth appears to involve collagenase activity. This mechanism was documented by Scandurro et al. (1993) who maintained hair follicles lacking dermal papilla, in the presence of bFGF. Under these conditions, pro-collagenase activity was initiated. This activation of collagenase has the potential to alter fibre growth by
mechanisms which affect the follicle collagen sheaths. In the wool follicle, the dermal papilla is separated from the mitotically active trichocytes of the follicle bulb by the basement membrane, which is continuous with the connective tissue sheath (Orwin and Woods 1982). The connective tissue sheath and basement membrane are formed from collagen fibres (Orwin 1988).

Fibroblast growth factor expression has been identified in the skin of sheep (Sutton et al. 1995). In addition, FGF has been identified within the basement membrane. Receptors for FGF have been located in the dermal papilla and hair bulb (Panaretto 1993). In vivo, elaboration of collagenase activity is likely to facilitate ingress of follicles through the dermis (Sutton et al. 1995), which suggests there is a role in the regulation of the follicle cycle rather than a direct effect on fibre growth. Expression of FGF in the skin of sheep suggests a role for this growth factor in the paracrine, rather than endocrine, regulation of fibre growth. The inhibition of fibre growth observed in this study may be suggestive of secondary messengers arising from FGF binding in the dermal papilla and affecting the activity of cells within the follicle bulb. Alternatively, the reduced fibre growth may be due to an enhancement of collagenase activity resulting in non-specific degradation of the follicle collagen sheaths.

The reduction in the fibre growth rate of isolated follicles maintained in the presence of EGF and TGFα is likely to occur through a similar mechanism as that presented for FGF. That is, EGF has also been shown to cause the enhancement of collagenase activity in follicles (Rogers et al. 1987). Transforming growth factor alpha binds to the EGF receptor and is, therefore, also likely to be capable of initiating synthesis of this protease (Wynn et al. 1989; Panaretto 1993). The data in the current study indicate that the order of potency of these three growth factors is bFGF > EGF > TGFα.

A further growth factor with the potential for inhibiting fibre growth is that of minoxidil, the active component of the hair restorative product REGAIM™. Although minoxidil has been shown to variously inhibit and stimulate fibre production by human hair follicles in vivo and in vitro (Philpott et al. 1990), a stimulatory effect was not achieved using isolated wool follicles. Evidence suggests that the mechanism by which minoxidil stimulates fibre growth is by regulating the follicle growth cycle. Application of minoxidil reduces the telogen phase of the follicle cycle (Frienkel et al. 1989) and maintains trichocytes in the follicle bulb in anagen (Baden et al. 1988); hence the increase in fibre length. A lack of minoxidil stimulation of wool growth by isolated follicles may be due to differences in the duration of the follicle cycle. For example, wool follicles may remain in anagen for up to eight years in sheep (Ryder and Stephenson 1968) which contrasts sharply with the much shorter duration seen with human hair follicles. The inhibitory effects on fibre growth in this study and by Philpott et al. (1990), may result from the toxic effects of minoxidil at high concentrations or from an as yet undetermined effect on fibre follicle activity.

The only growth factors to stimulate fibre production were the pituitary components ethanolamine and phosphoethanolamine. These components may provide exciting new mechanisms for the enhancement of fibre production, although the results were not statistically
significant and therefore require verification. Nonetheless, the potential exists for these components to have direct effects on fibre growth. Removal of the pituitary gland results in the cessation of fibre growth in vivo (Ferguson et al. 1965; Wallace 1979) and administration of pituitary extracts have been shown to stimulate wool production in vitro (Obana and Ito 1993) and in vivo (Ferguson et al. 1965; Wallace 1979). Phosphoethanolamine has growth promoting activities on rat tumour cell lines (Tsao et al. 1982) and, in concert with ethanolamine, is a potent stimulator of human keratinocyte growth (Hawley-Nelson et al. 1980; Breidahl et al. 1989).

Changes in the rate of wool growth in vivo can be independent of changes in plasma insulin concentrations (Bray et al. 1990). This supports the finding in this study that insulin does not affect the rate of wool growth by isolated follicles. Nonetheless, I believe that insulin does have a role in maintaining wool follicles in the active, anagen phase of growth. Westgate et al. (1993) reported that insulin was necessary for maintaining the anagen morphology of fibre follicles and the current study showed that follicle viability was reduced in the absence of insulin. However, these findings do not explain why elevated plasma insulin concentrations may be correlated with reduced (Wynn et al. 1988) or increased (Johnsson et al. 1985) wool production. It is likely that other growth factor controlled extra-follicular systems, such as nutrient partitioning, affect the efficacy of follicular control by insulin. In contrast to the apparent role of insulin, no role for insulin-like growth factor-I in regulating wool growth or the follicle cycle was supported by the current study.

I believe that these findings require that we now review our understanding of the mechanisms by which wool growth may be controlled. Indeed, it appears that control by regulating the growth phase of wool follicles is at least as likely as direct regulation of the fibre growth rate of follicles in anagen. Of the growth factors examined here, insulin, TGFα, EGF and bFGF each appear to have the potential to affect follicle activity.

In the current study, no effects on fibre growth of melatonin, prolactin or aqueous antler extract were observed. It may be that the time-frame was insufficient for their effects on fibre growth to be apparent. Within the few days over which the rate of fibre growth was measured, cellular effects on gene transcription or translation, receptor sensitivity or receptor number may have been initiated. However these may have had insufficient time for translation into measurable changes in wool production. In contrast, factors which inhibited wool production may have immediate direct effects which are rapidly and readily observable in a wool follicle culture system.

In conclusion, the study presented here defines appropriate conditions for the maintenance of isolated wool follicles in vitro and has shown that extra-follicular regulation of wool production occurs through the expression of endocrine growth factors, rather than modification of the wool follicle itself. Specific inhibitory proteins in ovine serum have been identified. Basic fibroblast growth factor, epidermal growth factor and transforming growth factor alpha have been presented as potential mediators of endocrine fibre growth regulation. I believe that the results from this
study support the hypothesis that endocrine growth factors contribute to the regulation of wool growth.

11.3 Potential for Future Research

The research in this thesis has led to the development of an in vitro model of wool growth. This now allows factors which may be involved in the regulation of wool production to be evaluated at the follicle level in the absence of whole-body influences. The following areas of research appear to have potential.

- The culture of isolated wool follicles may be used to identify whether different regulatory mechanisms exist for primary and secondary follicles. This could be undertaken by distinguishing between the types of follicle at isolation, on the basis of accessory structures and size, in some breeds such as the Drysdale. Any differential responses of the two follicle populations to growth factor challenges could then be evaluated.

- Similarly, the identification of primary and secondary follicles may allow an evaluation of the factors responsible for regulation of the follicle growth cycle in the two types of follicles. The current study suggests that there is a reduction in the proportion of follicles in anagen during winter. In future studies, identification of which follicle population is affected by season could be undertaken.

- It would be useful to structurally examine isolated follicles following culture with specific growth factors. This may enable further elucidation of the mechanisms by which wool growth is controlled. Conversion of isolated follicles from anagen to telogen or catagen following a growth factor challenge, may provide evidence that considerable regulation of wool growth occurs by controlling the phase of the wool growth cycle, rather than the rate of fibre extension.

- Wool follicle culture may also enable the identification of pathways involved in the initiation and maintenance of medulla formation. For example, the tissue culture conditions, particularly with respect to nutrients, could be modified. Changes in medulla volume may then signal the presence of factors important for the synthesis of keratin-filled cortical cells.

- Fibre growth and follicle viability in Dulbecco's modified Eagles Medium was significantly less than in William's Medium E. Use of the former culture medium, which provides minimal nutrient factors, therefore provides a follicle growth system by which potential stimulators of fibre growth can be examined.

- Cultured wool follicles could also be used to further study the seasonal effects of ovine serum on wool growth. The collection of serum at different times of the year and use of these samples to supplement the culture medium of follicles collected over a 12 month period, would provide a much clearer picture of the seasonality of extra-follicular regulators of wool growth.

- An important extension of the current study is the identification of factors in the two fractions of ovine serum which were identified as inhibitors of wool growth. Purification of these
components has the potential to provide exciting new leads into the mechanisms of wool growth regulation. The fractions could be purified using a variety of techniques, such as density gradient electrophoresis, isoelectric precipitation and solvent fractionation. Following purification, the individual components could be added to the culture medium to assess their potential for affecting wool production. Identification of active components could be undertaken, once purification was sufficiently high. Individual proteins could be characterised in a succession of approaches to ascertain their molecular weight, number of polypeptide chains, amino acid composition and amino acid sequence. This would then provide the identity of growth factors with the ability to inhibit wool growth in a season-dependent manner.

- The use of *in situ* PCR amplification for the identification of keratin genes in wool follicle sections was described for the first time in the current study. This provides the basis for developing techniques to amplify follicular mRNA *in situ*. Identification of the site of gene expression in wool follicle sections would enable considerable advances to be made in identifying key components in the biochemical pathways of wool growth regulation. While the current study suggests that responses to growth factors may not occur within three days, certainly changes in the transcription of genes may be identified using *in situ* PCR of mRNA.

In conclusion, the use of this *in vitro* model of wool growth has the potential to provide exciting new insights into the mechanisms that regulate wool growth and follicle activity. However, it must be remembered that this is a model system. I am well aware that findings developed using this technique require considerable validation, *in vivo*, prior to their establishment as fact. Nonetheless, to quote Fraser and Short (1960), "It is necessary, in research, to balance description, experimentation and speculation". I believe that this *in vitro* model of wool growth allows us to make further steps in speculating upon, experimenting with and drawing conclusions upon, factors with the potential to regulate wool growth *in vivo*. 
In life, the first thing you must do is decide what you really want. Weigh the costs and the results. Are the results worthy of the costs? Then make up your mind completely and go after your goal with all your might.

Alfred A. Montapert
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I would also like to thank a number of people in the UK who afforded me portions of their time. Mike Philpott and particularly Terence Kealy spent some time with me discussing the intricacies of fibre follicle culture. I also have cause to be grateful to Colin Jahoda for his enthusiastic discussions on follicle dermal papilla and for the chance to isolate some first hand. Thanks also to Colin and to Amanda Reynolds for their encouragement of my project. My thanks also go to Stewart Gilmour for introducing me to the magic of molecular biology and to Jon Hickford and Geraldine Rogers for practical advice about getting results from polymerase chain reaction (PCR) analysis. Thanks also to Geraldine for providing me with the keratin gene primers used in this study. I have received invaluable advice from Vicky Cameron that considerably lowered my learning curve during the adaptation of PCR for in situ analysis. Fractionation of ovine serum was undertaken at Life Technologies, Christchurch; I am very grateful for their generosity with respect to both time and equipment.

On a more personal note, I would never have considered undertaking this enterprise had it not been for my father, the late Bruce Winder. Throughout my life he always made me believe I could do anything, which has given me the courage to continue through many of the darkest days of this project. You were right Dad, thanks. I am also very grateful to my mother, Lois Witte, for being there for me and for sharing so many of the high and low points over so many years. Her positive attitude to life has encouraged me along the path many a time. Thanks also to my stepfather, Leon Witte, who has shared with me valuable insights into the realities of sheep and wool production on many occasions. The constant support of you both has made a real difference to me, thank you.

A final note of thanks to the friends I was able to lean on for support. Stephen Goldson has provided a solid sounding board throughout my post-graduate study and I have really appreciated his good advice, generous support and sailing diversions. Dave Mainwaring has also shared with me many of the later aspects of this study and I am grateful to him for the constancy of his support. Thanks also to Veronika Philips, Luisa Mattioli, Jacky Sargent and Mark Siddall for their friendship and their regular requests for updates which made procrastination ever more difficult.


Black J.L. and Reis P.J., (1979), Speculation on the control of nutrient partition between wool growth and other body functions, In: Physiological and Environmental Limitations to Wool Growth, Black J.L. and Reis P.J., editors, p.269, Armidale : University of New England Publishing Unit.


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(Abstract)


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REFERENCES


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Appendix A

A.1 Detailed Example of Analysis of Variance Program Format Using
GENSTAT 5 190

A.2 Output from GENSTAT 5 Statistical Package 191
A. Detailed Example of Analysis of Variance Program Format Using GENSTAT 5

" Directing commands and output to a file "
open '94-13.out'; c = 2; filetype = output : output 2

" Description of job "
" Results from growing follicles in culture
- from the data of EL94-13
NOT INCLUDING <30microns per day

Trt 1 = WE
Trt 2 = WE + 0.2mg/ml antler extract
Trt 3 = WE + 0.4mg/ml antler extract
Trt 4 = WE + 0.6mg/ml antler extract "

units [82]
" Reading data and doing any calculations or sorting
data occurs here (terminated by :) "
read [print = d,e,s] gr_rate

42 52 109 | 130 | 158 | 266 | 275 | 320 | 332 | 372 | 441 | 446
544 587 685 | 792 | 807 | 820 | 48 | 70 | 104 | 131 | 146 | 181
202 229 244 | 272 | 355 | 445 | 465 | 551 | 569 | 574 | 585 | 611
630 33 | 48 | 50 | 69 | 82 | 143 | 159 | 160 | 238 | 305 | 308
314 369 419 | 485 | 494 | 560 | 594 | 603 | 632 | 693 | 33 | 54
61 64 70 | 85 | 98 | 133 | 149 | 152 | 178 | 183 | 184
228 238 327 | 439 | 472 | 505 | 628 | 634 | 716 | 748 :

" Setting up factors "
factor [labels=lt('control','0.2mg','0.4mg','0.6mg')] Trt ;
!((18(1), 19(2), 21(3), 24(4)))

" Printing factor levels and raw and calculated variables as a check "
print Trt, gr_rate ; fieldwidth = 10; decimals = 0

" Setting up treatment structure "
treatments Trt
calc n = nlevels(Trt)
vari [n] r,m

" Doing ANOVA and F-probability tests "
for v = gr_rate
anova [orth = y; factorial = 5; contrasts = 9; fprob = y] v
akeep terms=Trt ; means = M ; rep = R ; var = V
aplot normal, histogram, fitted
equate news = r ; olds = R
equate news = m ; olds = M
calc sed = sqrt(V*(1/MEAN(element(r; 1)) + 1/element(r;!(2...n))))
calc dif = element(m;!(2...n)) - MEAN(element(m; 1))
calc t = dif/sed
calc pr = 1 - FPROMB(t**2;1;nobs(v)-n)
print dif,sed,pr
endfor
stop
A.2  **OUTPUT FROM GENSTAT 5 STATISTICAL PACKAGE**

Results from growing follicles in culture - from the data of EL94-13  
NOT INCLUDING < 30 microns per day  
Trt 1 = WE  
Trt 2 = WE + 0.2 mg/ml antler extract  
Trt 3 = WE + 0.4 mg/ml antler extract  
Trt 4 = WE + 0.6 mg/ml antler extract

### Data

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Minimum</th>
<th>Mean</th>
<th>Maximum</th>
<th>Values</th>
<th>Missing</th>
</tr>
</thead>
<tbody>
<tr>
<td>gr_rate</td>
<td>33.0</td>
<td>327.8</td>
<td>820.0</td>
<td>82</td>
<td>0</td>
</tr>
</tbody>
</table>

28

factor [labels = !t('control','0.2mg','0.4mg','0.6mg')] Trt ; 
!(18(1), 19(2), 21(3), 24(4))

31

print Trt, gr_rate ; fieldwidth = 10; decimals = 0

<table>
<thead>
<tr>
<th>Trt</th>
<th>gr_rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>42</td>
</tr>
<tr>
<td>control</td>
<td>52</td>
</tr>
<tr>
<td>control</td>
<td>109</td>
</tr>
<tr>
<td>control</td>
<td>130</td>
</tr>
<tr>
<td>control</td>
<td>158</td>
</tr>
<tr>
<td>control</td>
<td>266</td>
</tr>
<tr>
<td>control</td>
<td>275</td>
</tr>
<tr>
<td>control</td>
<td>320</td>
</tr>
<tr>
<td>control</td>
<td>332</td>
</tr>
<tr>
<td>control</td>
<td>372</td>
</tr>
<tr>
<td>control</td>
<td>441</td>
</tr>
<tr>
<td>control</td>
<td>446</td>
</tr>
<tr>
<td>control</td>
<td>544</td>
</tr>
<tr>
<td>control</td>
<td>587</td>
</tr>
<tr>
<td>control</td>
<td>685</td>
</tr>
<tr>
<td>control</td>
<td>792</td>
</tr>
<tr>
<td>control</td>
<td>807</td>
</tr>
<tr>
<td>control</td>
<td>820</td>
</tr>
<tr>
<td>0.2mg</td>
<td>48</td>
</tr>
<tr>
<td>0.2mg</td>
<td>70</td>
</tr>
<tr>
<td>0.2mg</td>
<td>104</td>
</tr>
<tr>
<td>0.2mg</td>
<td>131</td>
</tr>
<tr>
<td>0.2mg</td>
<td>146</td>
</tr>
</tbody>
</table>
33 treatments Trt
34
calc n = nlevels(Trt)
35
36 varl [n] r,m
37
38 for v = gr_rate
39    anova [orth = y; factorial = 5; contrasts = 9; fprob = y] v
40    akeep terms=Trt ; means = M ; rep = R ; var = V
41    aplot normal, histogram, fitted
42    equate news = r ; olds = R
43    equate news = m ; olds = M
44    calc sed = sqrt(V*(1/M( element(r;1)) + 1/element(r!1;(2...n))))
45    calc dif = element(m!1;(2...n)) - M( element(m;1))
46    calc t = dif/sed
47    calc pr = 1 - FPROB(t**2;1;obs(v)-n)
48    print dif,sed,pr
49    endfor
50
51........................................................................................................
**** Analysis of variance ****

Variate: gr_rate

Source of variation  d.f.  s.s.  m.s.  v.r.  F  pr.
Trt                    3  167494. 55831. 1.07 0.366
Residual               78  4062443. 52083.  
Total                  81  4229937.  

**** Tables of means ****

Variate: gr_rate

Grand mean 328.

<table>
<thead>
<tr>
<th>Trt</th>
<th>rep.</th>
<th>control</th>
<th>0.2mg</th>
<th>0.4mg</th>
<th>0.6mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>399.</td>
<td>337.</td>
<td>322.</td>
<td>272.</td>
</tr>
</tbody>
</table>

**** Standard errors of differences of means ****

Table rep. unequal
s.e.d. 76.1X min.rep
          71.2 max-min
          65.9X max.rep

(No comparisons in categories where s.e.d. marked with an X)

<table>
<thead>
<tr>
<th>dif</th>
<th>sed</th>
<th>pr</th>
</tr>
</thead>
<tbody>
<tr>
<td>-61.30</td>
<td>75.06</td>
<td>0.4166</td>
</tr>
<tr>
<td>-76.97</td>
<td>73.30</td>
<td>0.2970</td>
</tr>
<tr>
<td>-126.61</td>
<td>71.16</td>
<td>0.0791</td>
</tr>
</tbody>
</table>

Histogram of residual grouped by !(335.9,...,447.9)

Scale: 1 asterisk represents 1 unit.
Normal plot

expected Normal quantiles
****** End of job. Maximum of 19434 data units used at line 51 (618620 left)
Appendix B

B.1 Tissue fixation 197
B.2 Paraffin embedding 197
B.3 Deparaffinizing sections 197
B.4 Protease digestion 197
B.5 DNase digestion (negative control) 197
B.6 Postfixation 198
B.7 Polymerase chain reaction (PCR) 198
B.8 DIG detection 199
B.9 Eosin-Y staining 199
B.10 Mounting slides 199
Details of the *in situ* polymerase chain reaction technique provided here relates to the evaluation of follicle structure following maintenance *in vitro*, described in Chapter 4.

### B.1 Tissue Fixation

Individual follicles were fixed for two hours at 4°C in 10% formal saline. The fixative was prepared by diluting commercially available 40% formaldehyde 1:3 with phosphate buffered saline (PBS) and adjusting the pH to 7.2. The fixative was cooled to 4°C before use. Following fixation, follicles were washed in PBS twice for 10 min each at room temperature and prepared for paraffin embedding.

### B.2 Paraffin Embedding

Individual, fixed follicles were dehydrated by immersion for five minutes in each of 30%, 50%, 70%, 95% and 100% ethanol at room temperature, followed by two, 1 hour incubations in xylene. Follicles were submersed in molten paraffin wax at 65°C for 2 hours prior to embedding in a fresh solution of molten paraffin wax. Wax tissue blocks were stored at room temperature. The day before required, 5μm sections were cut and floated on a 40°C water bath containing ddH2O. Sections were then floated onto Polysine™ (Erie, U.K.) pre-coated microscope slides and contact with all portions of the section ensured by covering the slide with filter paper and pressing down. The small size of the tissue sections meant that numerous consecutive sections could be placed on one slide. Slides were dried at 40°C overnight.

### B.3 Deparaffinizing Sections

Dried tissue sections were cleared in xylene in a fume hood for 30 minutes at room temperature to remove the wax before being hydrated through an ethanol gradient series (2 minutes in each of 100%, 95%, 70%, 50% ethanol and ddH2O). Slides were then immersed in ddH2O at 37°C for at least 15 minutes.

### B.4 Protease Digestion

Slides containing the tissue sections were incubated in pepsin (1mg/ml in 0.2N HCl) for 30 minutes at 37°C. They were then washed in warmed (37°C) PBS for 10 minutes.

### B.5 DNase Digestion (Negative Control)

Selected slides were used as a negative control by digestion of DNA. These slides were incubated in 0.4U/μl DNase 1, RNase free (Boeringher Mannheim) in DNase buffer (0.01M Tris/HCl pH 7.5, 0.01M MgCl2) at 37°C for 5 minutes. They were then washed in PBS at 37°C for 10 minutes.
B.6 POSTFIXATION

Following digestion, slides were postfixed in 10% formal saline, pH 7.2, for 5 minutes at room temperature and washed with PBS (37°C) for at least 10 minutes.

B.7 POLYMERASE CHAIN REACTION (PCR)

A PCR reaction protocol was prepared based on that described by (Rogers et al. 1994). Twenty five microlitres of reaction mix was applied to each of four slides, with an additional 20μl mixed with sheep genomic DNA in a 1.5μl eppendorf tube overlaid with 20μl mineral oil as a positive control. The 3' and 5' keratin primers used in this reaction were designed and provided by Dr G.R.Rogers (Wool Research Organization of New Zealand, Canterbury, New Zealand) and are described in (Rogers et al. 1994). Briefly they encode portions of exon 7 of the KRT2.10 intermediate filament protein with an mRNA product of ≈ 1600bp following PCR. The nucleotide sequence of the primers was:

K2.10ex7 5'ATGGCCTGCTGCTCAAGGAG
K2.10D 5'GTGTTGTATTCTCAGAACACAGGG

The PCR reaction mix consisted of:

\( \text{ddH}_2\text{O} \quad 92.6\mu l \)
\( 10X \text{Taq buffer (with 1.5mM MgCl}_2, \text{Boehringer Mannheim)} \quad 13.0\mu l \)
\( 3' \text{ primer} \quad 13.0\mu l \)
\( 5' \text{ primer} \quad 6.5\mu l \)
\( \text{digoxigenin-UTP (vial 6, DIG kit, Boehringer Mannheim)} \quad 2.6\mu l \)
\( \text{dNTP's (Stock solution, 25mM, Boehringer Mannheim)} \quad 1.04\mu l \)
\( \text{Taq polymerase (Boehringer Mannheim)} \quad 1.3\mu l \)
\( \text{TOTAL} \quad 130\mu l \)

The 25μl aliquot for each slide was pipetted as a bead down the centre of a coverslip large enough to cover the entire area of tissue sections. Slides which had been equilibrated in PBS were blotted dry and inverted onto the coverslip using the technique described by (Simmons et al. 1989). Application of the slide onto the coverslip, rather than vice-versa, allows the solution to flow over the sections without air bubbles forming. The edges of the coverslip were then sealed with a bead of DPX mounting medium.

Four slides were placed in a stainless steel boat designed in our laboratory to fit an Autogene II thermal cycler. This cycler has the advantage, for this application, that temperature regulation is achieved within a waterbath which enables easy modification to a slide cycler. The positive control DNA tube was taped to the outside of the slide holder.

The thermal cycling conditions were 95°C, 65°C and 72°C (1 minute each) for 30 cycles followed by a final extension step at 72°C for 7 minutes.
A negative control was run without the inclusion of Taq polymerase.

**B.8 DIG DETECTION**

Following PCR, sections could be incubated overnight if required but were typically analyzed the same day. DPX mountant, which had dried during the PCR procedure was easily removed with tweezers and the coverslips washed off in warm PBS. DIGoxigenin (DIG) incorporated during primer elongation in the PCR reaction was detected following the protocol described by Boehringer Mannheim Ltd. Blocking reagent, DIG antibody, NBT and X-Phos were supplied by Boehringer Mannheim in their DIG detection kit.

Slides were washed in cleaning buffer (100mM maleic acid, 150mM NaCl, pH 7.5) and incubated in the same buffer containing 1% v/v blocking reagent (blocking buffer) for 30 minutes. Slides were then incubated in digoxigenin antibody (1:5000 dilution with blocking buffer) for 30 minutes and washed twice in cleaning buffer for 15 minutes. Slides were incubated in activation buffer (100mM Tris-HCl, pH 9.5, 100mM NaCl, 50mM MgCl₂) for two minutes before incubation overnight in the dark with NBT and X-Phos in activation buffer. When the reaction was complete, slides were washed with neutralizing buffer (10mM Tris-HCl, pH 8, 1mM EDTA) before eosin staining.

**B.9 EOSIN-Y STAINING**

Following PCR, all slides were stained with Eosin-Y, an orange/pink stain which does not obscure the blueish DIG colouration. The stain was prepared following the protocol of (Kiernan 1990), 2.5g eosin (CI 45380) is dissolved in 495ml ddH₂O and 5ml glacial acetic acid. It is possible to store this solution for a number of months and it can be filtered if bacterial growth is observed. Slides were stained in eosin for 30 seconds, then washed in running distilled water until no further colour was removed.

**B.10 MOUNTING SLIDES**

Following eosin staining, sections were dehydrated through an ethanol gradient series (50% for 10 minutes, 70% and 95% for 2 minutes each, 100% for 10 minutes). Sections were then placed in xylene for 20 minutes and air dried for 2 minutes prior to mounting with DPX. Mounted sections were examined using a light microscope.
APPENDIX C  PUBLISHED REPORTS OF THIS STUDY


DECLARATION

I hereby certify that the experimental work contained in this thesis was executed as described by the candidate under the direct supervision of Dr Roy Bickerstaffe.

R. Bickerstaffe

Dr Roy Bickerstaffe
Reader in Biochemistry
Animal and Veterinary Sciences Group
Lincoln University