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Madekurozwa, Mary-Catherine N. (1996) *Studies on the development and innervation of the bovine and caprine horn*. PhD thesis.

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**STUDIES ON THE DEVELOPMENT AND INNERVATION OF
THE BOVINE AND CAPRINE HORN**

by

Mary-Catherine N. Madekurozwa, BVSc, BSc. (Hons)

A thesis submitted for the Degree of
Doctor of Philosophy
in the Faculty of Veterinary Medicine
of the University of Glasgow

Research conducted in the Department of Veterinary Anatomy,
University of Glasgow.

November 1996.

ABSTRACT

The main objective of this study, as detailed in Chapter 2, was to investigate the nerves innervating the horn base in cattle and goats, paying particular attention to the contribution of the *N.sinuum frontaleum*. The study involved the gross dissection of twenty-five cattle, aged between 2 days and 4 years, as well as twenty-four goats aged between 10 hours and 6 years. In addition to the dissections, the neurotracer Fluorogold was applied to the cut horn bases of two calves and two 15-month-old heifers. Fluorogold was taken up by cut nerves and was then transported in a retrograde manner along the nerves. The inspection of nerve sections under the fluorescence microscope revealed Fluorogold granules in the *R.zygomaticotemporalis* of all four animals, as well as in the *N.infratrochlearis* of two animals and in the *N.frontalis* of a single animal. Fluorogold granules were not present in either the *N.sinuum frontaleum* or the *Nn.cervicales*. However, osmium-stained histological sections of the horn base revealed the presence of nerve fascicles, presumed to be branches of the *N.sinuum frontaleum*, in the cornual diverticular lining of cattle over 2 years of age and goats over 4 years of age. These results demonstrated that in cattle the *R.zygomaticotemporalis* is not the only nerve supplying the horn base. Furthermore, in mature cattle and goats the *N.sinuum frontaleum* needs to be blocked before the animal is dehorned.

The objective of Chapter 3 was to investigate the sensory receptors present in the horn bud of calves and kids. For this study immunohistochemistry was used in conjunction with transmission electron microscopy. Immunohistochemistry utilized neurofilament and S-100 antibodies which demonstrated nerve fibres and Schwann cell cytoplasm respectively. In this study numerous free nerve endings, known to be nociceptors, were identified in both calves and kids. In addition, bulbous corpuscles, Merkel cells and intraepidermal nerves were demonstrated. Ruffini corpuscles and perifollicular terminals were observed associated with hair follicles. The wide range of well-

developed sensory receptors identified suggest that the horn bud is a very sensitive area. These findings emphasize the need for local anaesthesia before disbudding calves and kids.

Chapter 4 involved the use of histochemistry, scanning electron microscopy and transmission electron microscopy to provide information on the development of the horn bud. A periderm layer covered the horn bud and adjacent skin of the bovine foetuses. The periderm covering the horn bud showed an intense reaction for glycogen, whereas only traces of glycogen were demonstrated in the adjacent skin. Furthermore between 104 and 125 days of gestation protrusions arose from the periderm cells in the horn bud region. Such protrusions were not seen in the adjacent skin. The information obtained has provided a baseline for future studies on the potential disruption of horn bud development as a means of eliminating the need for disbudding animals.

The objective of Chapter 5 was to study the development of the cornual process in calves and kids in order to determine whether or not a separate centre of ossification exists for the cornual process. Histology, radiography and Alizarin-red-S bone staining were used to study the formation of the bone underlying the horn bud in bovine foetuses aged between 58 and 246 days of gestation. Histological sections of the horn of post-natal calves and kids were also studied. A separate centre of ossification for the cornual process was not found in any of the pre-natal and post-natal calves studied. However a separate ossification centre for the tip of the cornual process was found in two kids.

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ACKNOWLEDGEMENT

I wish to express my sincere gratitude to my supervisors, Professor J.S. Boyd and Dr M. Purton, for having faith in my ability.

I would like to thank Iain MacMillan for technical assistance with the immunohistochemical and Fluorogold studies, as well as Alan Reid for help with the silver impregnation and Alizarin-red-S staining of bovine foetuses. Thanks are also due to Dr P. McNeil for allowing me to use her fluorescence microscope.

I would also like to thank Sheila Cranstoun and Jacqueline MacPherson for their help in the preparation of material for ultrastructural examination. I am grateful to Alan May for assistance with photography.

Lastly my thanks go to Olivia Hamudi, Johnston Ngugi, Fauziah Orthman, Mohd Azam Khan, Marielle Lorusso, Necla Gunduz and Kathleen Crawford for keeping me cheerful even in the bleakest of winters.

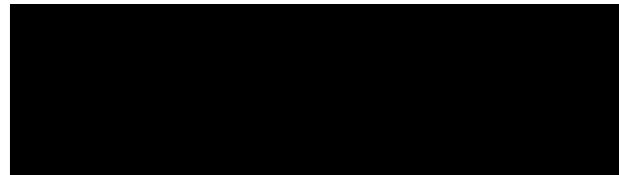
The financial support of the University of Zimbabwe Staff Development Programme is gratefully acknowledged.

DEDICATION

To my parents, Rae and Edgar, for their encouragement and inspiration in my formative years, and to my sisters Rachel, Theodora, Matilda, Bulelwa and Wezi for their love and support.

DECLARATION

I hereby declare that the work in this thesis was carried out by me in the Department of Veterinary Anatomy between December 1993 and November 1996. Any technical assistance that I received has been acknowledged.



Mary-Catherine N. Madekurozwa.

November 1996.

LIST OF ABBREVIATIONS

LM	=	Light Microscopy
SEM	=	Scanning Electron Microscopy
TEM	=	Transmission Electron Microscopy
A.	=	<i>Arteria</i>
M.	=	<i>Musculus</i>
N.	=	<i>Nervus</i>
Nn.	=	<i>Nervi</i>
R.	=	<i>Ramus or Rami</i>

CHAPTER 1

GENERAL CHAPTER.

GENERAL INTRODUCTION

Before dehorning cattle and goats, the nerves supplying the horn base need to be blocked. In the case of cattle the *R.zygomaticotemporalis* is blocked, whereas in goats both the *R.zygomaticotemporalis* and *N.infratrocchlearis* are blocked. However, despite blocking these nerves some animals exhibit signs of pain during dehorning. This suggests the presence of additional nerves supplying the horn base.

A review of the literature as detailed in Chapter 2 has revealed that remarkably few studies have been carried out on the nerves innervating the horn base in cattle and goats. It has been reported that, in addition to the *R.zygomaticotemporalis*, the *N.frontalis* (Blin, 1960; Lauwers and De Vos, 1966), *N.infratrocchlearis* (Butler, 1967), *N.sinuum frontarium* (Lauwers and De Vos, 1966) and *Nn.cervicales* (Taylor, 1955) occasionally innervate the horn base in cattle. In goats it has been reported that the *Nn.cervicales* may innervate the horn base in addition to the *R.zygomaticotemporalis* and *N.infratrocchlearis* (Linzell, 1964; Garrett, 1988; Thirkell et al., 1990).

Out of all these nerves, the *N.sinuum frontarium* has received the least attention. It is known that branches of the *N.sinuum frontarium* ramify throughout the mucosa of the *Sinus frontalis*, and, as the cornual diverticulum is an extension of the *Sinus frontalis*, it is assumed that branches from the *N.sinuum frontalis* extend into the cornual diverticulum. If this is the case the *N.sinuum frontarium* has to be blocked before dehorning mature cattle and goats. However, little is known of the occurrence of *N.sinuum frontarium* branches in the cornual diverticulum or the age at which such branches are found in the diverticulum. For this reason the main objective of Chapter 2 was to investigate the presence of nerves in the cornual diverticulum of cattle and goats ranging in maturity from newborn to adult. The investigation involved the use of gross dissections, endoscopy and the neurotracer, Fluorogold. In addition, the work carried out in Chapter 2 aimed to provide more information on the other nerves

innervating the horn base in cattle and goats.

Although the Animal (Anaesthetics) Act states that all domestic ruminants should be dehorned with the use of local anaesthesia, calves and kids are often disbudded without the use of local anaesthesia. This appears to be due to the assumption that young animals feel little pain during disbudding as the horn is poorly developed at this stage. However, there are several reports in the literature on the stress and apparent pain caused by disbudding without local anaesthesia. Although studies have been carried out on the stress reactions of calves and kids to disbudding, no studies appear to have been carried out on the sensory innervation of the horn bud. The objective of Chapter 3 was therefore to study the sensory receptors present in the horn bud of calves and kids, in order to find out whether the sensory innervation of the horn bud is poorly developed in the first four weeks after birth which is the period during which calves and kids are usually dehorned.

As dehorning appears to be a painful procedure, it would be desirable to avoid the process if possible. However, this is not practical in management systems where cattle and goats are intensively reared. In such systems dehorning is needed to prevent horn-induced injury to animals and human attendants. Dehorning could however be avoided if a method was devised to disrupt horn growth, which, like other ectodermally-derived structures, is probably dependent on epidermal-dermal interactions. However, before the epidermal-dermal interactions influencing horn development can be investigated it is necessary to study the normal development of the epidermal and dermal layers forming the horn. The objective of Chapter 4 was therefore to carry out a histological and ultrastructural study on the development of the horn bud in calves and kids. The results of this study will form a baseline for future studies on horn bud development and its possible disruption.

The theme of development was carried through to Chapter 5 where the development of the cornual process was investigated. It is widely accepted that the cornual process in cattle and goats arises as an outgrowth from the frontal bone. However, there are a few reports in the literature on the presence of a separate centre of ossification for the cornual process in both cattle and goats (Dove, 1935; George, 1956; Ganey et al., 1990). Despite these reports no studies on the pre-natal and post-natal development of the cornual process have been carried out. The objective of this final investigative chapter was therefore to study the development of the cornual process in pre-natal and post-natal animals using techniques known to accurately detect centres of ossification.

GENERAL MATERIALS AND METHODS

ANIMALS

Bovine foetuses of Aryshire and Friesian breeds were obtained from Glasgow and Paisley abattoirs. Foetal ages were estimated using crown-rump lengths and data from publications by Winters *et al.* (1942), Nichols (1944) and Warner (1958). The foetuses were used in studies involving histology, scanning electron microscopy, transmission electron microscopy, radiography and Alizarin-red-S bone staining. Details of these foetuses are shown in Tables 3.2, 4.1, 5.1, 5.2 and 5.3. Twenty-four goats of Scottish feral and New Zealand breeds, aged between 10 hours and 6 years, were used in gross anatomical (Table 2.4) and histological studies (Table 3.4). A total of twenty-five cattle of Friesian and Scottish Highland breeds, aged between 2 days and 4 years, were used in gross anatomical (Table 2.3), histological (Table 3.3) and ultrastructural studies. After sedation with xylazine, a lethal intravenous injection of sodium pentobarbitone (Euthatal: May and Baker, Dagenham) was used to euthanase the cattle and goats.

LIGHT MICROSCOPY

All tissue samples, from collection sites detailed later, were fixed in neutral buffered formalin for 5 days. The fixative was prepared as follows:

BUFFERED NEUTRAL FORMALIN

Formalin (40% W/V)	500ml.
Tap water	4500ml.
Sodium dihydrogen orthophosphate	20g.
Disodium hydrogen orthophosphate	32.5g.

The tissues were trimmed to 1cm square before decalcification in ethylene-diamine-tetra-acetic acid (EDTA). Completion of decalcification was confirmed by radiographing the tissues. The tissues were then processed in a Shandon Elliot automatic tissue processor using the following schedule:

70% alcohol	1 hour 30 minutes
Methylated spirits	1 hour 30 minutes
Methylated spirits	1 hour 30 minutes
Absolute alcohol	3 hours
Absolute alcohol	2 hours
Absolute alcohol	1 hour
Absolute alcohol:Chloroform (1:1)	1 hour
Chloroform:Histoclear (1:1)	2 hours
Histoclear	1 hour 30 minutes
Paraffin wax	3 hours
Paraffin wax	2 hours 30 minutes

The tissues were embedded in paraffin wax and mounted on blocks which were then trimmed to the level of the tissue. The blocks were then stored at 8°C overnight before being sectioned at 5µm with a Leitz rotary microtome. After mounting on slides the sections were stained using the following standard techniques:

Mayer's haematoxylin and eosin (H&E)

Masson's trichrome (MT)

Osmium tetroxide

Periodic-Acid-Schiff (PAS)

SCANNING ELECTRON MICROSCOPY

Tissues, from collection sites detailed later, were washed in sodium cacodylate to remove debris and blood. The tissues were then fixed in Karnovsky's fixative for a minimum of 24 hours. Karnovsky's fixative was made up as follows:

SOLUTION A

Paraformaldehyde	20g.
Distilled water	200ml.
1N sodium hydroxide	16 drops.

The solution was covered and placed in a 60°C oven overnight. When cool, solution A was added to solution B.

SOLUTION B

Sodium cacodylate	21.4g.
0,2 M hydrochloric acid	40ml.
25% glutaraldehyde	100ml.
Distilled water	660ml.

The tissues were then dehydrated in graded acetone as follows:

30 % acetone	4 hours
50 % acetone	4 hours
70 % acetone	12 hours
90 % acetone	2 hours
100% acetone	2 hours
100% acetone	12 hours

The tissues were then critically point dried in liquid carbon dioxide after which they were attached to aluminium stubs using silver paint. They were then allowed to dry for 30 minutes in an oven set to 37°C. The tissues were sputter coated with gold-palladium for 4 minutes before being examined in a Phillips 501 B scanning electron microscope set at 15 KV and using spot sizes 500 and 1000.

TRANSMISSION ELECTRON MICROSCOPY

1 mm square blocks of tissue were fixed in Karnovsky's fixative at 4°C for 3 hours. This was followed by immersion in 0.1 M. sodium cacodylate buffer for 1 hour. The tissues were then post-fixed for 1 hour in 1% osmium tetroxide buffered with 0.1 M. sodium cacodylate, before being rinsed in 3 changes of 0.1 M. sodium cacodylate buffer and processed as follows:

25% ethanol	10 minutes
50% ethanol	10 minutes
70% ethanol	10 minutes
90% ethanol	10 minutes
100% ethanol	45 minutes
Ethanol:Propylene oxide (1:1)	10 minutes
Propylene oxide	10 minutes
Propylene oxide	10 minutes
Araldite:Propylene oxide (1:1)	4 hours
Araldite:Propylene oxide (3:1)	12 hours
100% araldite	6 hours

The tissues were then embedded in capsules filled with Araldite, which were then polymerised at 60°C for 48 hours. Blocks were trimmed using an LKB

Pyramitone, with 2μm sections being cut and stained with toluidine blue to identify areas of interest. The toluidine blue solution was made up as follows:

TOLUIDINE BLUE SOLUTION

1% Sodium tetraborate	1g.
1% Toluidine Blue	1g.
Distilled water	100ml.

Ultrathin sections of the blocks were then cut on an LBK MKII ultramicrotome and collected in a boat containing 5% acetone. The resultant sections were attached to Athena 3.05 mm copper grids, and stained with uranyl acetate and lead citrate for 5 minutes each. The uranyl acetate and lead citrate solutions were made up as follows:

URANYL ACETATE

Uranyl acetate	2g.
Distilled water	100ml.

LEAD CITRATE

Lead citrate	1.33g.
Sodium citrate	1.76g.
Distilled water	30ml.
0.1M Sodium hydroxide	8ml.

The grids were examined using a JEM 100CX transmission electron microscope.

PHOTOGRAPHY**LIGHT MICROSCOPY**

A Leitz Laborlux S microscope connected to a Wild MPS 45 photoautomat unit was used for light microphotography. Ektachrome 64T film was used for colour photomicrographs, whereas Agfa PAN 35 mm film (12 ASA) was used for black and white micrographs. For black and white micrographs, Agfa-Gevaert Rapitome photographic paper was used. Processing of the micrographs was done in an Agfa-Gevaert Rapidoprint DD 3700 automatic processor.

SCANNING ELECTRON MICROSCOPY

Ilford FP4 120 (125 ASA) film in a Rollifex camera was used. Black and white micrographs were processed in an Agfa-Gevaert Rapidoprint DD 3700 automatic processor using Rapidoprint photographic paper.

TRANSMISSION ELECTRON MICROSCOPY

Ilford technical plates were used. The plates were developed in PQ universal developer and fixed in Ilford ilfospeed fixative. Black and white micrographs were prepared as for scanning electron microscopy.

CHAPTER 2

**THE INVESTIGATION OF HORN INNERVATION
IN THE BOVINE AND CAPRINE: A STUDY
USING
GROSS ANATOMICAL AND NEUROTRACER
TECHNIQUES**

INTRODUCTION

Before the introduction of intensive farming, cattle were not routinely dehorned except in the case of a fractured horn (Knowlson, 1834). With more intensive farming methods, dehorning became necessary to prevent horn-induced injuries to animals and human attendants (Clark, 1890; Hill, 1894; Vaughan and McDiarmid, 1951; Gendreau, 1953). Despite the advantages of dehorning cattle, many people objected to the procedure due to the pain inflicted on the animals. An anonymous letter to The Veterinary Record (1894) described dehorning as a "barbarous, unnecessary operation". Opposition to the perceived pain associated with dehorning led to the introduction in Britain of The Animals (Anaesthetics) Act in 1919. The act stated that dehorning could only be performed under general anaesthesia by a veterinarian. In America, where dehorning was still performed by laymen without the use of anaesthetic, fears of an introduction of the stringent legislation already in force in Britain prompted Emmerson (1933) to develop a practical method of dehorning animals with the use of local anaesthesia. After identifying the *R.zygomaticotemporalis*, a branch of the *N.ophthalmicus*, as the main sensory supply to the horn base, he was able to block this nerve pathway by injecting anaesthetic midway between the eye and horn base. This blocking technique developed by Emmerson was, however, found to be ineffective in some animals (Browne, 1938; Wheat, 1950; Mitchell, 1966^a), which suggested either the presence of additional nerves supplying the horn, or a variation in the branching pattern and course of the *R.zygomaticotemporalis*. There have been a few reports in the literature confirming the presence of additional nerves (*N.frontalis*, *N.infratrochlearis*, *N.sinuum frontalium* and the *Nn.cervicales*) supplying the horn. Blin (1960) reported observing a branch of the *N.frontalis* supplying the horn base in cattle, while Butler (1967) obtained complete desensitization of the horn base in Friesian and Hereford calves after blocking both the *R.zygomaticotemporalis*

and *N.infratrochlearis*. Lauwers and De Vos (1966) grossly traced the course of the *N.sinuum frontaleum*, which they described as originating from the *N.ophthalmicus* and terminating in the *Sinus frontalis* lining. They suggested the presence of *N.sinuum frontaleum* branches to the cornual process, but stated that these branches were very thin in most animals. Butler (1967) indicated that in addition to the *R.zygomaticotemporalis*, the horn in older animals may receive branches from the *N.auricularis magnus*. According to Taylor (1955) branches from both the *N.occipitalis major* and *N.auricularis magnus* occasionally reached the horn.

As in cattle management, goats are also dehorned to prevent horn-induced injuries to other goats and human attendants. In addition to this, goats are dehorned to decrease the amount of living space required by the goats and to prevent the animals from becoming trapped in fences (Spaulding, 1977). In cattle only the *R.zygomaticotemporalis* is routinely blocked before dehorning, whilst in goats both the *R.zygomaticotemporalis* and *N.infratrochlearis* are routinely blocked. Incomplete desensitization of the horn area after these nerves have been blocked has been reported in both cattle and goats (Browne, 1938; Wheat, 1950; Linzell, 1964). The aim of the present study was therefore to investigate the nerve innervation to the horn, paying particular attention to the *N.sinuum frontaleum* which has been observed innervating the cornual process in cattle (Lauwers and De Vos, 1966). To date no systematic studies have been carried out to determine the age at which the *N.sinuum frontaleum* contributes to the innervation of the horn, or the significance of the innervation in relation to innervation from the *R.zygomaticotemporalis*. No studies have been carried out in goats to determine whether or not the *N.sinuum frontaleum* contributes to the innervation of the horn. The present study aimed to determine the proportion of cattle and goats of different age groups in which the *N.sinuum frontaleum* innervated the cornual process. The sensory innervation of the horn base was

investigated using gross dissection, endoscopy, histological techniques and the neurotracer Fluorogold.

A REVIEW OF THE NERVES SUPPLYING THE HORN

Due to confusion over the terminology employed, it was felt that a brief review of the nerves supplying the horn would be helpful. A summary of the nerves supplying the horn in cattle and goats is shown in Tables 2.1 and 2.2. The innervation of the horn includes the following nerves:

R.ZYGOMATICOTEMPORALIS

Although *Nomina Anatomica Veterinaria* (1992) lists the *R.zygomaticotemporalis* as a branch of the *N.zygomaticus*, which is a division of the *N.maxillaris*, researchers studying the origin of the *R.zygomaticotemporalis* in ruminants list the nerve as a branch of the *N.ophthalmicus*. This is due to differences in the exit ports of the *N.maxillaris* and *N.ophthalmicus* from the cranial cavity (McClure and Garrett, 1966). In the dog, where the *N.maxillaris* and *N.ophthalmicus* have separate exits from the cranial cavity, the *N.zygomaticus* arises from the *N.maxillaris* (Evans, 1993). However, in ruminants, where the *N.ophthalmicus* and *N.maxillaris* leave the cranial cavity through a common opening, the *foramen orbitotundum*, the *R.zygomaticotemporalis* originates from the *N.ophthalmicus* (Mitchell, 1966^a; Godinho and Getty, 1971; Sisson and Grossman, 1976).

The *R.zygomaticotemporalis* has been given different names and origins by various authors. Some authors have referred to the *R.zygomaticotemporalis* as the *N.lacrimalis* (Lauwers and De Vos, 1966; Buttle *et al.*, 1986), whilst others have described the *R.zygomaticotemporalis* as being a branch of the *N.lacrimalis* (Vitums, 1954; Mitchell, 1966^{a,b}; Elmore, 1980; Constantinescu,

1991). Merlu (1953) referred to the *R.zygomaticotemporalis* as the *N.frontalis*.

The *R.zygomaticotemporalis* arises as two large fasciculi from the lateral side of the *N.ophthalmicus* on exiting from the *Foramen orbitotundum* (Sisson and Grossman, 1976). The two fasciculi travel over the dorsolateral surface of the orbit in contact with the medial border of the *M.rectus lateralis* before passing over the *A.ophthalmica externa* (Godinho and Getty, 1971). The two nerve fasciculi unite before the *R.zygomaticotemporalis* leaves the orbit. The united *R.zygomaticotemporalis* runs caudally through retro-orbital fat, before emerging from under the caudal border of the *M.frontoscutularis*. The *R.zygomaticotemporalis* courses ventral to the *Linea temporalis* where it is covered by the *M.frontalis* and skin (Sisson and Grossman, 1976). It gives off branches which unite with branches of the *N auriculopalpebralis* which is a branch of the *N. facialis* (Godinho and Getty, 1971; Sisson and Grossman, 1976). The *R.zygomaticotemporalis* divides into three or four *R.cornuales* which supply the caudolateral aspect of the horn (Lauwers and De Vos, 1966). Merlu (1953) observed the *R.zygomaticotemporalis* branching into three nerves close to the horn base. The first nerve divided into two branches which innervated the rostral aspect of the horn base. The second nerve coursed through a foramen in the cornual process to reach the mucosa of the cornual diverticulum in which it was distributed. The third nerve coursed caudally to supply the caudal horn and adjacent skin.

The *R.zygomaticotemporalis* in goats has a similar origin and course as in cattle (Sisson and Grossman, 1976). However, only in some animals is the *R.zygomaticotemporalis* composed of two fasciculi. The nerve courses dorsally on the medial surface of the *M.rectus lateralis* before penetrating the periorbita (Sisson and Grossman, 1976). The *R.zygomaticotemporalis* emerges from the orbit caudal to the *Processus supraorbitalis* and courses caudodorsally, through retro-orbital fat covered by the *M.frontalis* (Vitums, 1954). *R.cornuales* from the

R.zygomaticotemporalis supply the caudolateral aspects of the horn (Vitums, 1954; Thirkell *et al.*, 1990).

N.INFRATROCHLEARIS

Godinho and Getty (1971) traced the course of the *N.infratrochlearis* in five cattle of various breeds. They noted that in cattle the *N.infratrochlearis* was the continuation of the *N.nasociliaris* after the *N.ethmoidalis* had left the main branch. The *N.nasociliaris* is a medial branch of the *N.ophthalmicus*. The *N.infratrochlearis* runs upwards on the surface of the *M.rectus medialis* before dividing into two branches, which pass under the *M.obliquus dorsalis* (Godinho and Getty, 1971). The branches then course over the dorsal rim of the orbit one-third of the distance from the medial angle to the lateral angle of the eye before passing under the *M.orbicularis oculi*, and through the *M.frontalis* to supply the frontal skin in most animals and the rostral-lateral surface of the horn in a few animals (Sisson and Grossman, 1976). As it courses through the *M.frontalis* the *N.infratrochlearis* unites with branches from the *N.auriculopalpebralis* (Sisson and Grossman, 1976).

Butler (1967) carried out a series of dissections on nine cattle (three calves; one 18-month-old steer; two cows dehorned as calves and three horned cows). He noted that the *N.infratrochlearis* in the two dehorned cows was much smaller than in the horned animals.

Vitums (1954) described the course of the *N.infratrochlearis* in eight goats. The *N.infratrochlearis* divided into two branches as it passed over the orbital rim. The smaller medial branch, referred to as the frontal branch, innervated the skin of the frontal region. The lateral *R.cornualis* divided into two branches which supplied the rostromedial and caudomedial surfaces of the horn. The description of the course of the *N.infratrochlearis* by Getty (1975) differed from that given by Vitums (1954). Getty (1975) reported that after its

origin from the *N.nasociliaris*, the *N.infratroclearis* divided into two branches on the *M.rectus medialis*. These branches coursed under the *M.obliquus dorsalis* before ramifying into several small branches, some of which coursed over the dorsal orbital rim. The branches supplied the rostral-lateral surface of the horn.

N.FRONTALIS

Lauwers and De Vos (1966) studied the origin of the *N.frontalis* in thirty-two cattle ranging in age from 20 months to 7 years. The *N.frontalis* was seen to originate either directly from the *N.ophthalmicus* or from the medial fasciculus of the *R.zygomaticotemporalis*. In two animals both variations were present. In addition to the origins noted by Lauwers and De Vos (1966), Godinho and Getty (1971) observed the *N.frontalis* originating on a common trunk with the *N.sinuum frontarium*. The *N.frontalis* then coursed dorsally in the orbit, crossing the *A.ophthalmica externa* and passing under the lacrimal gland before coursing over the dorsal rim of the orbit. Diesem (1968) noted that in some animals the *N.frontalis* emerged from the *Foramen supraorbitale* as the *N.supraorbitalis*. In the majority of cases the *N.frontalis* supplies the upper eyelid and adjacent areas, where it unites with branches of the *N.auriculopalpebralis* (Godinho and Getty, 1971). However, Lauwers and De Vos (1966) observed branches of the *N.frontalis* supplying the horn in six out of the thirty-two cattle studied. In five out of six animals a thick branch of the *N.frontalis* pierced the base of the *Processus zygomaticus* and coursed under the *M.frontalis* along the *Linea temporalis*. Along its course the branch gave off small nerves which united with branches of the *R.zygomaticotemporalis*. The main branch of the *N.frontalis* ramified into small *R.cornuales* close to the horn base. In the sixth animal the *N.frontalis* coursed dorsally over the rim of the orbit and then along the *Linea temporalis*. The *N.frontalis* united with the *R.zygomaticotemporalis* midway along the *Linea temporalis*. Blin (1960) found that the *N.frontalis* divided into three branches one

of which innervated the horn base.

The *N.frontalis* originated directly from the *N.ophthalmicus* in five out of ten goats studied by Godinho and Getty (1971). In three animals the *N.frontalis* originated from the *N.ophthalmicus* on a common trunk with the *N.sinuum frontale*. In one of the animals the nerve originated on a common trunk with the *N.lacrimalis*, whilst in the last animal the nerve originated from the *R.zygomaticotemporalis*. The *N.frontalis* then crossed the *A.ophthalmica externa*, coursed along the lateral border of the *M.rectus dorsalis* before passing under the lacrimal gland. The nerve divided into two branches which passed dorsally over the orbital rim, before ramifying into smaller branches which supplied the upper eyelid. There do not appear to be any reports of the *N.frontalis* supplying the horn in goats.

N.SINUUM FRONTALIUM

In most of the cattle dissected by Godinho and Getty (1971), the *N.sinuum frontale* arose on a common trunk with the *N.frontalis*. The origin of the *N.sinuum frontale* from the *R.zygomaticotemporalis* was rare. Lauwers and De Vos (1966) observed the *N.sinuum frontale* originating from either the *N.frontalis* or *R.zygomaticotemporalis*, as well as directly from the *N.ophthalmicus*. The *N.sinuum frontale* then coursed dorsally between the *M.rectus dorsalis* and *M.rectus lateralis* before entering the *Sinus frontalis* through a foramen in the medial wall of the orbit (Sisson and Grossman, 1976). Either before, or soon after entering the foramen the nerve divides into two branches, one of which courses through the periosteum of the cranium before eventually entering the *Sinus frontalis caudalis* where it has a tortuous course through the bone enclosing the *Sinus frontalis* (Lauwers and De Vos, 1966). Branches of the *N.sinuum frontale* were seen entering the cornual diverticulum of the horn in five out of thirty-two animals. It was noted that these

branches were very small and were probably of little significance in horn innervation.

Lauwers and De Vos (1966) described the course of an unnamed nerve which originated either from the *N.sinuum frontale* or from the *N.ophthalmicus*. The nerve was described as a transition between the *N.sinuum frontale* and the *R.zygomaticotemporalis*, because it coursed through a groove in the *Linea temporalis* before giving off branches which entered the *Sinus frontalis caudalis*. In four animals, branches from this unnamed nerve were traced to the horn. Lauwers and De Vos (1966) stated that even though these branches were clearly visible, they were very small in comparison to branches from the *R.zygomaticotemporalis*.

In goats the *N.sinuum frontale* originates on a common trunk with the *N.frontalis* from the *N.ophthalmicus* (Godinho and Getty, 1971). The nerve then courses dorsally on the surface of the *M.rectus dorsalis* before entering a foramen in the medial wall of the orbit. Branches of the *N.sinuum frontale* are distributed to the *Sinus frontalis* mucosa (Sisson and Grossman, 1976). There do not appear to be any published reports of branches of the *N.sinuum frontale* innervating the horn in goats.

Nn.CERVICALES

Taylor (1955) has reported that both the *N.auricularis magnus* (branch of the first cervical nerve) and the *N.occipitalis major* (branch of the second cervical nerve) could supply the horn in cattle. Butler (1967) suggested that the *N.occipitalis major* could supply the horn in mature cattle where the horn base had expanded caudally towards the neck, as in an 18-month-old steer he dissected. Garrett (1988) has also reported branches of the *N.occipitalis major* innervating the horn in some cattle and goats. In addition, Thirkell *et al.* (1990) were able to trace branches of the *N.occipitalis major* and *N.auricularis*

magnus.

ANATOMICAL BASIS FOR DEHORNING

CATTLE

In cattle the *R.zygomaticotemporalis* is often the only nerve blocked before dehorning (Gabel, 1964; Horney, 1966). The procedure for blocking the *R.zygomaticotemporalis* was introduced by Emmerson (1933) who marked the blocking site by drawing an imaginary line from the centre of the orbit to the lateral aspect of the horn base. The *R.zygomaticotemporalis* was blocked at a point midway along this imaginary line on the *Linea temporalis*. Later, Browne (1938) modified the blocking technique, used by Emmerson (1933), by blocking the *R.zygomaticotemporalis* 2.5cm from the horn base along the *Linea temporalis*. In addition, a second injection, 1cm cranial to the first, was required in large animals to block nerves which branched off before the first injection site. Despite this modified blocking procedure, failures were noted by Wheat (1950) who suggested that they were due to the *R.zygomaticotemporalis* having a more superficial course in some animals. He also noted that the distance between the pathway of the *R.zygomaticotemporalis* and the *Linea temporalis* varied between animals. A new blocking procedure was therefore devised in which the *R.zygomaticotemporalis* was blocked 4 to 4.5cm cranial to the horn base, 1 to 2cm ventral to the *Linea temporalis*. A similar injection site was recommended by Merlu (1953).

Since then the basic blocking procedure has had several modifications. Mitchell (1966^a) and Horney (1966) used techniques in which the *R.zygomaticotemporalis* was blocked midway between the lateral canthus and the horn base at a site ventral to the *Linea temporalis*. Mitchell (1966^a) noted that

if the blocking site was moved closer to the horn the block would be unsuccessful due to the branching of the *R.zygomaticotemporalis*. Tazewell and Greenough (1950) recommended blocking the *R.zygomaticotemporalis* one third of the distance from the lateral canthus of the eye to the horn. In calves the *R.zygomaticotemporalis* is blocked midway between the eye and base of the ear (Blin, 1960). Elmore (1980) and Skarda (1986) blocked the *R.zygomaticotemporalis* 2.5 to 4 cm from the horn base.

In addition to the *R.zygomaticotemporalis*, Butler (1967) found that the *N.infratrochlearis* had to be blocked in order to obtain complete desensitization of the horn. The *R.zygomaticotemporalis* was blocked one third of the distance from the horn base to the lateral canthus of the eye, whilst the *N.infratrochlearis* was blocked along the rostrodorsal edge of the orbit. In one of the animals dissected, Butler (1967) had noted a branch of the *N.ocipitalis major* ending close to the horn base. He stated that the *N.ocipitalis major* could be blocked on the dorsal midline of the neck at a site level with the caudal base of the ear. The blocking sites used by various researchers are shown in Figure 2.1.

GOATS

In goats the *R.zygomaticotemporalis* is blocked at the caudal aspect of the *Processus supraorbitalis* whilst the *N.infratrochlearis* is blocked at the rostromedial margin of the orbit (Vitums, 1954; Thirkell *et al.*, 1990) or midway between the medial canthus of the eye and the medial aspect of the horn base (Skarda, 1986). Linzell (1964) used the former procedure on a herd of one hundred goats and found it successful in most cases, but noted that some animals required an additional injection between the horns. Linzell (1964) did not describe the exact site of this injection, nor did he name the nerve supplying this area. Thirkell *et al.* (1990) have observed branches of the *N.ocipitalis major* and *N.auricularis magnus* innervating the caudal aspect of the horn base in

some animals. They recommended the injection of local anaesthetic in the caudal horn region to completely desensitize the horn.

The procedures in the literature (Mitchell, 1966^b; Bowen, 1977; Taylor, 1979; Papworth, 1981; Buttle *et al.*, 1986; Gray, 1986; Taylor, 1991) describing the blocking of the *R.zygomaticotemporalis* and *N.infratrochlearis* in goats are all based on the procedure used by Vitums (1954), as shown in Figure 2.2, and contain no additional information.

Table 2.1. A summary of nerves innervating the horn in cattle.

Author	Zygomatico-temporalis	Infra-trochlearis	Frontalis	Sinuum frontarium	Auricularis magnus	Occipitalis major
Emmerson, 1933	+++					
Tazewell & Greenough, 1950	+++					
Wheat, 1950	+++					
Gendreau, 1953	+++					
Merlu, 1953	+++					
Taylor, 1955	+++				+	+
Blin, 1960	++		++			
Gabel, 1964	+++					
Lauwers & De Vos, 1966	+++		+	+		
Mitchell, 1966a	+++					
Butler, 1967	++	++				+
Godinho & Getty, 1971	+++	++				
Sisson & Grossman, 1976	+++	++				
Elmore, 1980	+++					
Ashdown & Done, 1984	+++					
Skarda, 1986	+++					+
Dyce et al., 1996	+++	+	+			
Garrett, 1988	+++					++
Habel, 1989	+++		+			
Constantinescu 1991	+++					

Table 2.2. A summary of nerves innervating the horn in goats.

Author	Zygomatico-temporalis	Infra-trochlearis	Frontalis	Sinuum frontarium	Auricularis magnus	Occipitalis major
Vitums, 1954	++	++				
Linzell, 1964	++	++				
Mitchell, 1966b	++	++				
Butler, 1967	++	++				
Getty, 1975	++	++				
Bowen, 1977	++	++				
Taylor, 1979	++	++				
Elmore, 1981	++	++				
Ashdown & Done, 1984	++	++				
Buttle et al., 1986	++	++				
De Lahunta & Habel, 1986	++	++				
Gray, 1986	++	++				
Garrett, 1988	+++	++				+
Habel, 1989	++	++				
Thirkell et al., 1990	++	++			+	+
Taylor, 1991	++	++				
Dyce et al., 1996	++	+				

+++ = Indicates the main nerve supplying the horn.

++ = Indicates a slightly less important nerve or equal contribution from two nerves.

+ = Indicates a minor nerve supply.

MATERIALS AND METHODS

ANIMALS

Twenty-five cattle and Twenty-four goats varying in age from 10 hours to 6 years were used in this study. Accurate dates of birth for the goats and most of the cattle were obtained from farm records. Details of the cattle and goats used in the present study are shown in Tables 2.3 and 2.4.

GROSS ANATOMICAL PROCEDURES

DISSECTION OF NERVES SUPPLYING THE HORN BASE

The *R.cornuale*s of the *R.zygomaticotemporalis* were exposed by removing the skin covering the *Fossa temporalis*. To trace the *R.zygomaticotemporalis* to its origin, the head was cut in the median plane, after which the *Processus frontalis* and the *Processus zygomaticus* were removed. The *Os mandibula*, larynx and tongue were then removed. The *Os basisphenoidale* was removed to expose the trigeminal trunk.

The course of the *N.infratrocLEARIS* was followed after removal of the skin, *M.orbicularis oculi* and *M.frontalis* from the *Os frontale*. The origin of *N.infratrocLEARIS* was revealed by loosening the connective tissue attaching the periorbita to the orbital wall. The eye ball was then pulled laterally to expose the *N.infratrocLEARIS* and *N.nasociliaris*. The origin of the *N.frontalis* and *N.sinuum frontale* was revealed after removal of the rostral half of the *Os frontale* which resulted in the exposure of the dorsal periorbita.

Table 2.3. Details of cattle dissected.

Group	Age	Breed	Sex
0-6 weeks	2 days	Friesian	male
	1 week	Friesian	male
	2 weeks	Friesian	male
	2 weeks	Friesian	male
	3 weeks	Friesian	male
	3 weeks	Friesian	male
	4 weeks	Friesian	male
	4 weeks	Friesian	male
	5 weeks	Friesian	male
	6 weeks	Friesian	male
12-18 months	12-18 months	Friesian	female
	12-18 months	Friesian	female
	12-18 months	Friesian	female
	12-18 months	Friesian	female
	12-18 months	Friesian	female
	12-18 months	Friesian	female
	12-18 months	Friesian	female
	15 months	Friesian	female
	15 months	Friesian	female
2-4 years	2 years	Friesian	male
	2 years	Highland	*
	3 years	Highland	*
	3 years	Friesian	male
	3 years	Friesian	male
	4 years	Friesian	male

* = Sex unknown because only the heads were obtained from Glasgow abattoir.

Table 2.4. Details of goats dissected.

Group	Age	Breed	Sex
0-8 weeks	10 hours	feral cross	male
	2 weeks	feral cross	male
	3 weeks	feral cross	male
	3 weeks	feral cross	male
	4 weeks	feral cross	male
	4 weeks	feral cross	male
	6 weeks	feral cross	male
	8 weeks	feral cross	male
	8 weeks	feral cross	male
	8 weeks	feral cross	male
4-12 months	4 months	feral cross	female
	4 months	feral cross	female
	6 months	feral cross	female
	6 months	feral cross	female
	6 months	feral cross	female
	12 months	feral cross	female
2-6 years	2 years	feral cross	female
	3 years	feral cross	female
	3 years	feral cross	female
	3 years	feral cross	female
	4 years	feral cross	female
	5 years	N.Zealand	male
	6 years	N.Zealand	male
	6 years	N.Zealand	male

Feral cross = Scottish feral crossed with a New Zealand breed

N.Zealand = A New Zealand breed

SINUS ENDOSCOPY

Fibreoptic endoscopy was used to examine the lining of the cornual diverticulum. Endoscopy allowed a close inspection of a wide area of the *Sinus frontalis caudalis* and cornual diverticulum without the need to remove the upper plate of the *Os frontale*, which would have damaged the nerves in the lining. The upper third of the horn was sawn off to create an endoscopic portal. The *Sinus*

frontalis was then thoroughly lavaged with water to remove debris and blood. A Pentak FG 28B endoscope, with an attached camera, was used to examine the *Sinus frontalis caudalis* and cornual diverticulum. The camera was loaded with Kodak Ektachrome 160T film.

HISTOLOGICAL PROCEDURES

OSMIUM TETROXIDE

Osmium tetroxide was used to demonstrate the myelin sheaths of nerves in the horn dermis, cornual process and cornual diverticular lining. Osmium tetroxide is reduced by the lipids in myelin to metallic osmium which stains the myelin sheaths black.

0.5 cm. thick cross sections of the horn base were cut with a bandsaw as shown in Figure 2.3. The tissues were then fixed in buffered neutral formalin for 5 days, after which they were decalcified in ethylene diamine-tetra-acetic acid. The tissues were then cut into 2 x 2 cm. blocks which were placed into an aqueous 0.5% osmium tetroxide solution for 1 week. The blocks were then thoroughly washed to remove excess osmium tetroxide. After dehydration and paraffin-impregnation in a Shandon Elliot tissue processor, 5 μ m. sections were cut and mounted on glass slides. The sections were then dried on a hot tray. The slides were placed in HistoClear for 10 minutes to remove the paraffin wax. Cover slips were then placed on the slides. The slides were examined under the light microscope to locate nerve fascicles. The locations of the nerves in the dermis, bone and diverticular lining were mapped on drawings of cross sections of the horn base. All structures shown in the schematic illustrations were measured with an ocular micrometer to ensure that the structures were in correct proportion to each other.

TRACING THE NERVES INNERVATING THE HORN USING THE RETROGRADE AXONAL TRACER FLUOROGOLD

Introduction

The large number of animals incompletely desensitized after the routine *R.zygomaticotemporalis* block, as well as the conflicting reports on the innervation of the horn, show that the sole use of gross dissections to investigate the complete innervation of the horn is unreliable. This is due to the need for careful dissection in order to preserve all the nerves supplying the horn. The use of the neurotracer Fluorogold provides a means of labelling all the nerves innervating the horn. In recent years neurotracers have been used in conjunction with gross dissections to investigate the innervation of organs in the dog (Chibuzo and Cummings, 1981, 1982; Hudson, 1990), monkey (Chien *et al.*, 1994) and rat (Stathakis *et al.*, 1994), but to date neurotracers have not been used to trace nerves in domestic ruminants.

Literature review

Neurotracers which are transported in a retrograde direction have been used to study the origins and connections of axons in the central and peripheral nervous systems for many years, with horseradish peroxidase being one of the most commonly used. The main advantage of horseradish peroxidase was its high sensitivity, especially when conjugated to wheat germ agglutinin which facilitated its uptake by axons (Weinberg and Van Eyck, 1991). However, several problems were encountered when horseradish peroxidase was used. Firstly, horseradish peroxidase was capable of crossing plasma membranes (Mesulam, 1978). This resulted in a loss of horseradish peroxidase when the interval between injecting the neurotracer and sacrificing the experimental animal was

prolonged. Secondly, the demonstration of horseradish peroxidase in tissues required careful histochemical processing (Morrell *et al.*, 1981). Thirdly, most of the chromogens used in horseradish peroxidase were shown to be carcinogenic (Mesulam, 1978).

The introduction of a new neurotracer, Fluorogold, by Schmued and Fallon (1986) overcame many of the problems associated with horseradish peroxidase histochemistry. Fluorogold is a fluorescent retrograde axonal tracer which is supplied as a golden-yellow crystalline dye (Ng *et al.*, 1992). It is composed of two fluorescent components, one of which has been identified as the antibiotic hydroxystilbamidine (Wessendorf, 1991). The chemical composition of the second component, which is responsible for most of the fluorescence, is known only to the chemical company producing Fluorogold.

The major advantage of Fluorogold is that it does not cross plasma membranes and so is retained permanently in nerve cell bodies irrespective of survival periods (Schmued and Fallon, 1986). Because of this inability to cross plasma membranes, Fluorogold has to be applied to cut or crushed nerves in order to introduce it into nerves during experiments involving the peripheral nervous system (Baranowski *et al.*, 1992; Christian *et al.*, 1993). In studies of afferent pathways within the central nervous system, Fluorogold is injected into a small area using pressure or iontophoresis (Pieribone and Aston-Jones, 1988). After uptake, Fluorogold is transported retrogradely through the axons towards the cell bodies in lysosomes (Schmued *et al.*, 1989; Wessendorf, 1991).

Fluorogold does not require any histochemical processing. Sections for the demonstration of Fluorogold can be cut from frozen, paraffin-embedded or plastic-impregnated tissue (Schmued *et al.*, 1989). The intense gold fluorescence characteristic of Fluorogold is visible under the fluorescence microscope using wide band ultraviolet excitation (Christian *et al.*, 1993).

Fluorogold has been used in association with immunohistochemistry without any effect on the intensity of the fluorescence (Ju *et al.*, 1989; Sauer and Oertel, 1994; Allen and Cechetto, 1995). Fluorogold has also been used in conjunction with other neurotracers, such as Fast Blue (Hirai and Jones, 1988; Audinat *et al.*, 1988), Diamino Yellow (Takada *et al.*, 1987; Sahibzada *et al.*, 1987), and horseradish peroxidase (Kruse *et al.*, 1993), as well as viral axonal tracers (Papka *et al.*, 1995). Recently Fluorogold has been combined with *in situ* hybridization histochemistry (Cheung and Hammer, 1995).

Materials and methods

Animals

Two 15-month-old heifers and two male calves aged 5 weeks and 6 weeks (Figure 2.4) were used. The heifers had well-formed horns with well-developed cornual diverticuli. The calves' horn buds ranged in diameter from 2.5cm to 3cm and in height from 1cm to 1.5cm.

Experimental procedure

The animals were sedated with xylazine prior to induction with ketamine. General anaesthesia was maintained with halothane (Figure 2.5). Once the animals had reached a sufficiently deep level of anaesthesia, the area surrounding the horns was clipped and cleaned (Figure 2.6). The right horn of both heifers was resected at the base using large dehorning shears. Disbudding shears were used to disbud the two calves (Figure 2.7). The disbudding shears removed the whole horn bud, a thin rim of skin and the developing cornual process (Figure 2.8 A & B). Haemorrhage was controlled using haemostatic forceps (Figure 2.9). Once the haemorrhage had been controlled Fluorogold,

dissolved in sterile distilled water, was applied from a syringe to the horn base through a 25 gauge hypodermic needle (Figure 2.10). Concentrations of Fluorogold ranging from 2% to 8% were used¹. In the 6-week-old calf and in one of the heifers excessive haemorrhage diluted the Fluorogold solution. The Fluorogold was left on the horn for 30 minutes before the left horn was removed. Fluorogold was not applied to the left horn base which served as a control.

Recovery from surgery was uneventful with the animals showing no signs of irritation to the application of Fluorogold. After four to six weeks the animals were euthanased with a lethal intravenous injection of sodium pentobarbitone.

Histological processing

The entire length of the *R.zygomaticotemporalis*, *N.infratrochlearis*, *N.frontalis*, *N.sinuum frontarium*, and *Nn.cervicales* were dissected out and fixed in 10% buffered neutral formalin for 24 hours. The tissues were then cut in half longitudinally. One half of the nerve was placed in 15% sucrose for 24 hours and then sectioned at 18 to 20 μ m on a freezing microtome. The other half of the nerve was processed in an histokinette and embedded in paraffin wax. The paraffin embedded tissues were sectioned at 10 μ m. The paraffin sections were dried in an incubator (37°C) and dewaxed in xylene before being mounted using an ultraviolet-free, xylene-based mountant. The sections cut on the freezing microtome were dried in an incubator set at 37°C, dehydrated in graded alcohols, cleared in xylene (Histoclear decreases fluorescent emission) and then mounted in ultraviolet-free, xylene-based mountant.

¹ Since Fluorogold had not been used on domestic ruminants before, it was necessary to try a wide range of concentrations in order to determine the concentration at which Fluorogold uptake was optimum.

Viewing and photography

Sections were viewed with an Olympus BH-2 epifluorescence microscope using an ultraviolet excitation filter. A PM-10 AK automatic exposure camera was used to take photomicrographs using a Fuji 400 ASA daylight film. Exposure times varied from 2 to 3 minutes.

RESULTS

DISSECTION OF NERVES SUPPLYING THE HORN IN CATTLE

R.ZYGOMATICOTEMPORALIS

2 days- 4 weeks of age

In this age group the horn was innervated solely by *R.cornuales* from the *R.zygomaticotemporalis*. The lateral fasciculus of the *R.zygomaticotemporalis* was the first branch of the *N.ophthalmicus* once it had passed through the *Foramen orbitotundum*. The medial fasciculus arose from the *N.ophthalmicus* rostral to the origin of the lateral fasciculus. The medial fasciculus was about a third of the thickness of the lateral fasciculus. The two fasciculi ran dorsally along the *M.rectus lateralis* and united before the *R.zygomaticotemporalis* penetrated the periorbita surrounding the eye. The united *R.zygomaticotemporalis* then passed caudally on the medial aspect of the *Processus zygomaticus* of the *Os frontale*. The *R.zygomaticotemporalis* split into two thick *R.cornuales* at the rostral end of the *Fossa temporalis*. The dorsal *R.cornualis* passed caudally over the dorsal surface of the *M.frontoscutularis* and then under the *M.frontalis* before innervating the rostral surface of the horn. In the 1-week-old calf, a branch from this dorsal *R.cornualis* coursed medially onto the crown of the head where it ramified into several branches. The ventral *R.cornualis* divided into three

branches midway between the eye and horn. Two of the branches supplied the caudolateral aspects of the horn. One of these branches curved around the caudal part of the horn to innervate the medial surface of the horn. The third branch innervated the *M.frontoscutularis*.

A variation in the course of the *R.zygomaticotemporalis* was observed in one of the 3-week-old calves (Figure 2.11). In this animal the medial and lateral fasciculi of the *R.zygomaticotemporalis* did not unite within the periorbita. Instead, the lateral fasciculus emerged from the orbit, coursed along the *Fossa temporalis* and then divided into several *R.cornuales* which were distributed to the caudolateral aspect of the horn. A branch from the lateral fasciculus curved around the caudal edge of the horn bud and then coursed medially over the crown of the head. The medial fasciculus of the *R.zygomaticotemporalis* left the orbit through a foramen in the dorsolateral aspect of the *Os frontale*, 0.7 cm. from the orbital rim. This nerve coursed caudally under the *M.frontalis*. Approximately one-third of the distance between the orbit and the horn the medial fasciculus divided into two branches. The lateral branch divided into several branches which innervated the rostral-lateral surface of the horn. A few of these branches united with branches from the lateral fasciculus of the *R.zygomaticotemporalis*. The medial branch of the medial fasciculus coursed caudally and divided into several branches which innervated the rostral-medial surface of the horn as well as the adjacent *M.frontalis*. This deviation of the medial fasciculus of the *R.zygomaticotemporalis* was only observed on the left side of the head. The course of the *R.zygomaticotemporalis* on the right side of the head was as initially described.

In one of the 4-week-old calves dissected, a *R.cornualis* left the *R.zygomaticotemporalis* as it emerged from the caudal edge of the *Processus zygomaticus* of the *Os frontale*. The *R.cornualis* coursed dorsal to the *R.zygomaticotemporalis* to supply the rostral-medial aspect of the horn. The main

trunk of the *R.zygomaticotemporalis* divided into two branches approximately midway along the *Linea temporalis*. The branches supplied the rostral, lateral and caudal regions of the horn.

The various branching patterns observed in the calves dissected are shown in Figure 2.12 (A-C).

12-18 months old

In all the animals studied in this age group, the *R.zygomaticotemporalis* was the only nerve supplying the horn base. The origin and course of the *R.zygomaticotemporalis* within the periorbita was as described in the 2-day to 4-week age group. The *R.zygomaticotemporalis* emerged from the periorbita and coursed caudally through retro-orbital fat in the *Fossa temporalis*. Approximately midway between the lateral canthus of the eye and the horn base the nerve split into dorsal and ventral branches. The dorsal branch divided into at least three branches. One or more branches coursed around the rostral edge of the horn sending short branches into the connective tissue enclosing the cornual process. The remaining branches terminated in the lateral part of the horn base. The ventral branch of the *R.zygomaticotemporalis* sent a small branch to innervate the *M.frontoscutularis*, whilst the main nerve trunk curved around the caudal aspect of the horn base to supply caudal and medial regions of the horn.

2-4 years old

The *R.zygomaticotemporalis* was the main nerve supplying the horn in all the animals studied. The *R.zygomaticotemporalis* branched approximately midway between the eye and the horn base into several branches which supplied the rostral, lateral and caudal aspects of the horn base (Figures 2.13 & 2.14A). In one of the 2-year-old animals, the *R.zygomaticotemporalis* divided into

R.cornuales one third of the distance between the eye and horn. The *R.cornuales* were distributed to the rostral, lateral and caudal surfaces of the horn. Short anastomosing branches linked the *R.cornuales* as they coursed towards the horn.

In one of the 3-year-old animals a *R.cornualis* left the *R.zygomaticotemporalis* as it emerged from the orbit. The *R.cornualis* innervated the caudal and medial regions of the horn (Figure 2.14B).

N.INFRATROCHLEARIS

2 days- 4 weeks of age

The *N.nasociliaris* was seen as the continuation of the *N.ophthalmicus* after the *R.zygomaticotemporalis* and *N.frontalis* had been given off. The *N.nasociliaris* traversed the medial surface of the *M.rectus medialis* before terminating in two branches, the *N.infratrochlearis* and *N.ethmoidalis*. The *N.ethmoidalis* entered a foramen in the medial orbital wall whilst the *N.infratrochlearis* coursed dorsally along the caudal edge of the *M.rectus medialis*. Midway along its course the *N.infratrochlearis* divided into two branches of unequal thickness which then passed over the orbital rim and through the *M.orbicularis oculi* close to the medial canthus. The branches then split into several small nerves which innervated the medial frontal region. No branches were seen extending to the horn.

12-18 months old

The origin and course of the *N.infratrochlearis* within the periorbita was as described in the calves. The *N.infratrochlearis* split into two or three thick nerves which coursed over the medial and central aspects of the orbital rim and then through the *M.orbicularis oculi*. The branches ramified into several smaller

branches which then terminated in the *M.frontalis* in most animals.

In one animal the most lateral of the three branches of the *N.infratrochlearis* coursed through the *M.frontalis* to innervate the rostral horn base. The middle branch innervated the medial aspect of the horn, whilst the medial branch supplied the medial frontal region (Figure 2.15). This branching pattern was seen on the right side of the head. On the left side the *N.infratrochlearis* branched into two nerves as it coursed over the medial orbit. The lateral branch divided into two nerves which innervated the lateral and rostral horn base (Figure 2.16). The medial branch of the *N.infratrochlearis* innervated the medial frontal region.

2-4 years old

The *N.infratrochlearis* had an origin and course similar to the other age groups. However, in one of the 3-year-old bulls the lateral branch of the *N.infratrochlearis* supplied the medial aspect of the horn, whilst the medial branch innervated the area between the horns (Figure 2.13). This pattern of innervation was found on both sides of the head.

N.FRONTALIS

2 days- 4 weeks of age

The *N.frontalis* arose from the *N.ophthalmicus* on a common trunk with the *N.sinuum frontale*, rostral to the origin of the medial fasciculus of the *R.zygomaticotemporalis*. The *N.frontalis* then coursed dorsally between the *M.rectus dorsalis* and the *M.rectus lateralis* before giving off two to three branches which were distributed in the connective tissue forming the periorbita. The *N.frontalis* then passed under the lacrimal gland where it gave off a thin *N.lacrimalis*. In one of the 3-week-old calves the *N.frontalis* divided into two

branches of approximately equal size after giving off the small *N.lacrimalis*. The two branches then coursed over the dorsal orbital rim. In the remainder of the calves a single *N.frontalis* coursed over the dorsal orbital rim and then divided into several branches which innervated the lateral frontal region.

In all the calves dissected the *N.frontalis* was much thinner than the *N.infratroclearis*. No branches from the *N.frontalis* could be traced as far as the horn.

12-18 months old

The origin of the *N.frontalis* was as described in the calves. The *N.frontalis* coursed over the lateral part of the orbital rim, through the *M.orbicularis oculi*, where it divided into two small branches which ramified over the lateral region of the *M.frontalis*. In one of the 15-month-old heifers, the right lateral branch of the *N.frontalis* divided midway along the *Linea temporalis*. The dorsal branch continued along the *Linea temporalis*, eventually terminating in the rostral cut-edge of the horn base. The ventral branch ramified over the *M.frontoscutularis* (Figure 2.17). On the left side of the head the *N.frontalis* innervated the upper eyelid and the lateral frontal region; no branches reached the horn base. Likewise, in the remainder of the animals dissected no branches from the *N.frontalis* could be traced to the horn base.

2-4 years old

In this age group the *N.frontalis* innervated the lateral region of the *M.frontalis*. No branches from the *N.frontalis* could be traced to the horn base.

N.SINUUM FRONTALIUM

In all the animals studied the *N.sinuum frontale* originated on a common trunk with the *N.frontalis*, and then coursed medially across the *M.rectus dorsalis* before dividing into several small branches, one of which entered a foramen in the caudomedial wall of the orbit. The other branches were distributed in the periosteum of the bone of the orbit.

Nn.CERVICALES

In all the animals studied no branches from the *N.auricularis magnus* or *N.occipitalis major* could be traced to the horn base. Small branches from the *N.occipitalis major* ended on the caudomedial aspect of the ear.

DISSECTION OF NERVES SUPPLYING THE HORN IN GOATS

R.ZYGOMATICOTEMPORALIS

10 hours-8 weeks of age

In most of the kids the *R.zygomaticotemporalis* was composed of two fasciculi which arose from the *N.ophthalmicus*. The nerves coursed along the dorsal edge of the *M.rectus dorsalis* and sometimes united before exiting the periorbita. In eight kids studied, the *R.zygomaticotemporalis* was seen as a single nerve running ventral to the *Linea temporalis*. Close to the horn base the *R.zygomaticotemporalis* divided into several *R.cornuales* which were distributed to the caudolateral surfaces of the horn (Figure 2.18A). In some kids *R.cornuales* also innervated the rostral horn area (Figure 2.18B). In two kids aged 3 and 8 weeks, the *R.zygomaticotemporalis* emerged from the orbit as two fasciculi which ran caudally along the *Fossa temporalis*. The dorsal fasciculus divided

into at least two *R.cornuales* midway between the eye and the horn. These *R.cornuales* were distributed to the caudolateral aspects of the horn (Figure 2.18C). Branches from the ventral fasciculus of the *R.zygomaticotemporalis* innervated the *M.frontoscutularis*.

4-12 months old

The *R.zygomaticotemporalis* arose as a single or double fasciculi from the *N.ophthalmicus*. The fasciculi united within the periorbita in most cases. The *R.zygomaticotemporalis* emerged from the orbit and ran caudally to the horn base. As in the previous age group a branch was given off which supplied the *M.frontoscutularis*. The *R.zygomaticotemporalis* divided into two thin *R.cornuales* close to the horn base. The *R.cornuales* innervated the caudolateral horn base (Figure 2.18A), as well as the rostral horn base in some cases (Figure 2.18B).

2-6 years old

As before the *R.zygomaticotemporalis* arose as a single or double nerve from the *N.ophthalmicus*. The *R.cornuales* of the *R.zygomaticotemporalis* were very thin in adult animals, and were distributed to the caudolateral surfaces of the horn base (Figure 2.19). In a 5-year-old male and a 3-year-old female, two *R.zygomaticotemporalis* fasciculi emerged from the periorbita and were seen coursing ventral to the *Linea temporalis*. The dorsal fasciculus divided into branches which innervated the rostral aspect of the horn. The ventral fasciculus ramified into several branches which innervated the caudolateral surface of the horn (Figure 2.18D). The various branching patterns of the *R.zygomaticotemporalis* in all the goats studied are shown in Figure 2.18, A-D.

N.INFRATROCHLEARIS

10 hours- 8 weeks of age

At the rostral region of the *M.rectus medialis*, the *N.nasociliaris* divided into the *N.ethmoidalis* and *N.infratrochlearis*. The branching pattern of the *N.infratrochlearis* varied between animals. In most of the goats studied the *N.infratrochlearis* emerged from the medial aspect of the periorbita as a single nerve. Under the *M.orbicularis oculi* the *N.infratrochlearis* divided into two branches which coursed dorsomedially over the rim of the orbit. One or both branches innervated the horn base (Figure 2.20 A & B). In two kids, aged 10 hours and 6 weeks, the *N.infratrochlearis* coursed over the orbital rim as a single nerve which ramified into *R.cornuales* close to the horn (Figure 2.20D). In the 10 hours to eight week age group the *R.zygomaticotemporalis* and *N.infratrochlearis* were similar in size.

4-12 months old

The *N.infratrochlearis* had one or two thick branches which supplied the rostromedial aspect of the horn. In one of the 4-month-old goats the two *R.cornuales* of the *N.infratrochlearis* ramified into numerous small nerves which were distributed to the rostromedial horn as well as to the adjacent *M.frontalis* (Figure 2.20A). In the other animals studied, the branching of the *N.infratrochlearis* was not as extensive as in the 4-month-old goat. In this age group the *N.infratrochlearis* appeared to be much thicker than the *R.zygomaticotemporalis*.

2-6 years old

The *R.cornuales* of the *N.infratrochlearis* were larger than the *R.cornuales*

of the *R.zygomaticotemporalis* (Figure 2.19). In most animals the *N.infratrochlearis* divided into three branches beneath the *M.orbicularis oculi*. In five animals branches from the *N.infratrochlearis* coursed to the horn base where they divided into numerous branches which innervated the rostromedial surfaces of the horn base (Figure 2.20C). In a 4-year-old female, the *N.infratrochlearis* divided into three branches below the *M.orbicularis oculi*. However, only one branch could be traced to the horn base (Figure 2.20E).

N.FRONTALIS

In all the goats studied, the *N.frontalis* was a very thin nerve which coursed over the lateral aspect of the orbital rim and then innervated the lateral region of the *M.frontalis*. None of the branches of the *N.frontalis* could be traced to the horn base.

N.SINUUM FRONTALIUM

10 hours- 8 weeks of age

The *N.sinuum frontale* arose from the *N.ophthalmicus* rostral to the origin of the *N.frontalis*. In this age group the nerve was very thin. The *N.sinuum frontale* exited the orbit through a foramen in the rostromedial aspect of the orbit.

4-12 months old

As in the previous age group, the *N.sinuum frontale* was a very thin nerve. However in this age group one or two branches from the *N.sinuum frontale* were seen ending in the periosteum lining the orbit. The *N.sinuum frontale* entered the *Sinus frontalis* through a foramen in the craniomedial wall of the orbit.

2-6 years old

By 6 years of age, the *N.sinuum frontale* had become a substantial nerve. The nerve was approximately the same size as the *R.zygomaticotemporalis*. The *N.sinuum frontale* entered the *Sinus frontalis* through a foramen in the rostromedial orbit. Several nerves left the *N.sinuum frontale* before it entered the foramen.

Nn.CERVICALES

No branches from the *Nn.cervicales* could be traced as far as the horn base in any of the animals studied.

ENDOSCOPY

CATTLE

1-4 years old

In the Friesian cattle, the cornual diverticulum was not transected by any bony shelves. Thin-walled canals containing large blood vessels were occasionally seen in the wall of the cornual diverticulum. A few small nerves were seen in the lining of the cornual diverticulum, but no single large nerve was ever seen. In one of the 3-year-old animals, thin threads of nerves were seen in the *Sinus frontalis caudalis*, but none could be traced to the cornual diverticulum.

In the two Highland cattle studied, the cornual diverticulum was traversed by a series of thin anastomosing bony shelves which were lined by tortuous small blood vessels and accompanying thread-like structures which were assumed to be nerves. Large canals containing blood vessels coursed through

the walls of the more central regions of the bony shelves.

GOATS

1-6 years old

In the three large-horned male goats, aged between 5 and 6 years, several bony shelves divided the cornual diverticulum into several compartments. Nerves were clearly visible in the cornual lining of the bony shelves. In the female goats, which had small horns, no nerves were visible in the *Sinus frontalis caudalis* or in the cornual diverticulum, although blood vessels were clearly visible in both regions.

OSMIUM TETROXIDE-STAINED NERVES IN THE HORN

CATTLE

2 days- 4 weeks of age

In this age group the cornual diverticulum of the *Sinus frontalis* had not developed.

12-18 months old

Several nerves were present in the reticular dermis. Very few connective tissue papillae were seen, but those which were present extended deep into the cornual process which was still composed of immature woven bone with thin interlocking trabeculae separated by wide areas containing loose connective tissue. No nerves were identified in the cornual diverticular lining.

2-4 years old

At 2 years of age, large nerve fascicles were present in the deep dermis as well as in wide connective tissue papillae which extended into the cornual process. The cornual process was composed of compact bone formed by Haversian systems. The cornual diverticulum was a single compartment containing a thick loose connective tissue layer below the lining epithelium. In the rostral part of the compartment of one of the 2-year-old bulls, was a single nerve fascicle unaccompanied by blood vessels (Figure 2.21). In the other 2-year-old a few small nerves were seen in the walls of blood vessels.

In the 4-year-old animal, an irregular-shaped cornual diverticulum extended into the cornual process (Figure 2.22). The cornual process surrounding the cornual diverticulum was thick in comparison to the caprine cornual process. The cornual diverticular lining contained several blood vessels ranging in size from 40-80 μm . Small nerve fascicles (10-50 μm) were associated with these blood vessels. A canal containing approximately twelve nerve fascicles was found in the compact bone of the cornual process. The largest nerve fascicle within this canal was 140 μm in size. In comparison the nerve fascicles in the dermis of the horn ranged in size from 110-170 μm .

GOATS10 hours-8 weeks of age

In this age group the *Sinus frontalis* had not extended into the cornual process.

4-12 months old

At 4 months of age nerve fascicles were present in the deep dermis of the horn close to the periosteum. In one of the 4-month-old kids no connective tissue papillae were seen extending into the cornual process, whilst in the other kid numerous connective tissue papillae were observed extending a short distance into the bone. In both kids a thick periosteum separated the bone from the dermal layer of the horn. In addition, the cornual process in both animals was composed of immature bone made up of bony trabeculae separated by wide areas containing loose connective tissue. No large blood vessels or nerve fascicles were seen within the cornual process. Although the *Sinus frontalis* had extended into the cornual process by 4 months of age, no nerve fascicles were observed in the cornual diverticular lining.

In the 6-month-old goats many nerve fascicles were found in the reticular and deep dermal regions, as well as in connective tissue papillae which extended into the cornual process. The cornual process was formed by mature bone composed of Haversian systems. In all three 6-month-old goats studied, the cornual diverticulum was divided into two compartments by a bony shelf. In one of these animals, the rostral compartment of the cornual diverticulum contained a plexus of blood vessels within the cornual diverticular lining. The blood vessels were a combination of thick-walled arterioles and thin-walled venules. No isolated nerve fascicles were observed in the lining, although there were several small bundles present in the walls of the larger blood vessels. In the other 6-month-old animals studied, extensive vascular plexi were not observed. In these animals a few small nerve fascicles were observed in the walls of the large blood vessels located in the connective tissue layer of the cornual diverticulum.

2-6 years old

In one of the 3-year-old animals, nerve fascicles were located in the deep dermis as well as in the long connective tissue papillae which penetrated the cornual process. The cornual process was composed of mature compact bone with irregularly-shaped Haversian canals. Within the Haversian canals were nerve fascicles, many of which were larger than the nerves found in the dermis. In this animal the cornual diverticulum was a single undivided compartment. The cornual diverticular lining contained a few thick-walled blood vessels, but nerve fascicles were absent.

In the 4-year-old animal, several connective tissue papillae extended into the cornual process. At the tips of these papillae were large nerve fascicles. Smaller nerve fascicles were observed in the deep dermis. The cornual process was composed of compact bone with large nerve fascicles within the Haversian canals. The cornual diverticular lining contained large blood vessels and a few nerve fascicles. Connective tissue papillae from the cornual diverticular lining extended deep into the cornual process, almost penetrating the full thickness of the bone. These traversing canals contained large nerve fascicles.

In one of the 6-year-old bucks the cornual diverticulum at the horn base was divided into three compartments by thin shelves of bone (Figure 2.23). The rostral compartment was the largest. Nerve fascicles were found throughout the lining of the cornual diverticulum. In most cases the nerve fascicles were approximately the same size as the accompanying blood vessels. One of the compartments had a canal running through its outer wall. Coursing through the canal were several large nerve fascicles (120-430 μ m.) and blood vessels. Most of the nerve fascicles were associated with blood vessels, but in a few areas clusters of nerves were seen independent of blood vessels. In addition to the nerve fascicles in the sinus lining, numerous nerves were present in the compact

bone within the central canals of the Haversian systems. These nerve fascicles were much larger than those in the sinus lining.

In the other 6-year-old buck, most of the dermal nerve fascicles were located in wide connective tissue papillae which projected into the cornual process. The rest of the dermis had a sparse scattering of small nerves. The cornual process was composed of compact bone. Located between the Haversian systems were large areas containing loose connective tissue and large nerve fascicles. The nerve bundles in these areas were at least three times the size of the nerve fascicles found in the connective tissue papillae. The cornual diverticulum was divided by a series of shelves into six compartments. Nerve fascicles, similar in size to those seen within the cornual process, were found in the cornual diverticular lining. These large nerve bundles accompanied similar-sized blood vessels.

In both 6-year-old animals, the size and number of nerve fascicles within the cornual process far exceeded those found in the dermis.

TRACING THE NERVES INNERVATING THE HORN IN CATTLE USING FLUOROGOLD

In frozen sections Fluorogold was seen as bright yellow fluorescent granules, which were occasionally clumped (Figure 2.24). Although most of the granules were located in the centre of the axons, a few granules were located along the margins of the axons. The connective tissue forming the perineurium and endoneurium of the nerve, as well as the red blood cells, had a dull blue-green fluorescence, whilst the axons lacked fluorescence. The Fluorogold granules were thus easily recognized even at low magnification.

In paraffin sections the connective tissue enclosing the nerves, as well as

the red blood cells, had a bright fluorescence which made identification of Fluorogold granules difficult. The fluorescence of the Fluorogold granules was dull compared to the granules seen in frozen sections. Clumps of Fluorogold granules were not seen in paraffin sections. Due to the difficulty of observing Fluorogold granules in paraffin sections, only the results obtained from frozen sections are reported in this study.

CALVES

Fluorogold granules were noted in the *R.zygomaticotemporalis* and *N.infratrochlearis* of both calves. These Fluorogold granules were found in the periorbital section of both the *R.zygomaticotemporalis* and *N.infratrochlearis*. The *R.zygomaticotemporalis* of the 5-week-old calf appeared to contain more Fluorogold granules than the 6-week-old calf. Fewer granules were apparent in the *N.infratrochlearis* of both animals. None of the nerves from the left side of the head contained Fluorogold granules. A subjective assessment of Fluorogold granules observed in the *R.zygomaticotemporalis*, *N.infratrochlearis* and *N.frontalis* is shown in Table 2.5.

15-MONTH-OLD HEIFERS

In one of the animals numerous Fluorogold granules were present in the part of the *R.zygomaticotemporalis* which was located in the *Fossa temporalis*. In the other animal fewer Fluorogold granules were seen in the *R.zygomaticotemporalis*. The *R.zygomaticotemporalis* supplying the control horn base contained a few Fluorogold granules (Figure 2.25A).

In both animals a moderate number of granules were present in the extraorbital section of the *N.infratrochlearis*. In one of the animals a few granules were present in the extraorbital region of the *N.frontalis* (Figure 2.25B).

No granules were seen in either the *N.sinuum frontaleum* or in the *Nn.cervicales* of either animal. A summary of these results is shown in Table 2.5.

Table 2.5. Summary of the occurrence of Fluorogold in frozen sections of the *Nn.cervicales* and branches of the *N.ophthalmicus*.

Age	Horn*	Nerves					<i>Nn.cervicales</i>
		<i>Zygomatico-temporalis</i>	<i>Infra-trochlearis</i>	<i>Frontalis</i>	<i>Sinuum frontaleum</i>		
5 weeks	right	++	+	-	-	-	-
	left	-	-	-	-	-	-
6 weeks	right	+	+	-	-	-	-
	left	-	-	-	-	-	-
15 months	right	+++	++	-	-	-	-
	left	+	-	-	-	-	-
15 months	right	++	++	+	-	-	-
	left	+	-	-	-	-	-

++ = Moderate number of small granules.

+ = Occasional small granules.

- = No Fluorogold granules seen.

* = In all cases Fluorogold was applied to the right horn.

DISCUSSION

CATTLE

R.ZYGOMATICOTEMPORALIS

In all the cattle dissected the *R.zygomaticotemporalis* arose as two fasciculi from the *N.ophthalmicus*. In all but one animal the two fasciculi united before leaving the periorbita. The origin and course of the *R.zygomaticotemporalis* is in agreement with observations by Sisson and Grossman (1976), as well as Getty and Godinho (1971). In twenty-one of the animals dissected, once the *R.zygomaticotemporalis* had emerged from the periorbita it branched midway along the *Linea temporalis*. This branching pattern is the standard pattern noted in the literature and is the basis of the standard cornual block, in which anaesthetic is injected midway between the eye and horn base (Mitchell, 1966^a; Elmore, 1980). However, in the present study it was found that in one out of twenty-five animals the *R.zygomaticotemporalis* branched a third of the distance between the eye and horn base. In addition, in two of the twenty-five animals a *R.cornualis* left the *R.zygomaticotemporalis* immediately caudal to the *Processus zygomaticus*. In the three cases cited, infiltration of anaesthesia midway between the eye and horn would have resulted in an incomplete desensitization of the horn base. In the first case the blocking procedure recommended by Tazewell and Greenough (1950), in which the *R.zygomaticotemporalis* is blocked one-third of the distance between the horn and eye, would have desensitized the horn base. In the latter two cases only anaesthesia immediately caudal to the *Processus zygomaticus* would have successfully desensitized the horn base.

As well as variations in the branching pattern of the *R.zygomaticotemporalis* between individuals, variations also occurred in the

branching of the *R.zygomaticotemporalis* within individuals. In one of the 3-week-old calves dissected, the lateral and medial fasciculi of the *R.zygomaticotemporalis* on the left side of the head did not unite. In this animal the medial fasciculus exited the orbit through a canal caudodorsal to the orbital rim before coursing caudally over the *Os frontale* to reach the horn base. This variation in the course of the medial fasciculus was only found in one out of twenty-five animals, and only occurred on one side of the head. A standard cornual block would have failed to desensitize the horn in this case especially as the medial fasciculus anastomosed with branches of the lateral fasciculus, thus influencing the innervation of the rostral, medial and lateral aspects of the horn. Morisse *et al.* (1995) reported a partial anaesthetic failure in 40% of calves disbudded. They suggested that one of the possible reasons for the anaesthetic failure was a variation in the nerves supplying the horn. Lauwers and De Vos (1966) reported variations in the course of the medial fasciculus which followed unusual routes before uniting with the lateral fasciculus. The unusual course of the medial fasciculus noted in the present study differed from the cases reported by Lauwers and De Vos (1966) in that in the current study the medial fasciculus remained independent of the lateral fasciculus throughout its course, only contributing a few branches to the lateral fasciculus close to the horn base. Lauwers and De Vos (1966) reported that in three out of thirty-two cattle studied, a branch from the medial fasciculus of the *R.zygomaticotemporalis* coursed through the periosteum of the *os temporale*. In the first animal the branch joined the *R.zygomaticotemporalis* midway along the *Linea temporalis*. In the second animal the nerves united close to the horn base, whilst in the third animal the branch penetrated the *Linea temporalis* and coursed on the surface of the *Os frontale* before joining the *R.zygomaticotemporalis*. In the cases reported by Lauwers and De Vos (1966), variations were seen in the course of the medial fasciculus as it ran ventral to the *Linea temporalis*. In the present study the

variation in the course of the medial fasciculus occurred whilst it was still within the orbit.

Despite the variations seen in the course of the *R.zygomaticotemporalis* in the present study, this nerve was the main nerve supplying the horn base in the four animals in which the Fluorogold, was applied. Compared to the other nerves in which Fluorogold granules were visualized, the *R.zygomaticotemporalis* appeared to contain the most granules. However, it was noted that the number of Fluorogold granules observed in the four animals was influenced by the amount of haemorrhage present during the dehorning procedure. In one of the 15-month-old heifers and in the 6-week-old calf, haemorrhage diluted the Fluorogold applied to the cut horn base. Fewer Fluorogold granules were seen in the nerves of these two animals.

In the two heifers, but not in the calves, a few Fluorogold granules were found in the *R.zygomaticotemporalis* supplying the horn base on which Fluorogold had not been applied. This suggests that some branches of the *R.zygomaticotemporalis* cross over the crown of the head to innervate the opposite horn base or the skin surrounding the horn base.

N.INFRATROCHLEARIS

The origin and course of the *N.infratrochlearis* is in agreement with reports in the literature (Godinho and Getty, 1971; Sisson and Grossman, 1976). In the present study, branches of the *N.infratrochlearis* could be traced grossly to the horn base in only two animals. However, in all the animals used in the Fluorogold study, granules were found in the *N.infratrochlearis*, which indicated that branches of the *N.infratrochlearis* were cut during dehorning. This latter finding is in agreement with the observations made by Sisson and Grossman (1976), who listed the *N.infratrochlearis* as one of the main nerves innervating the horn base. Likewise, Butler (1967) reported that the *N.infratrochlearis* had to

be blocked in six out of nine animals dehorned. Although branches of the *N.infratrocLEARIS* may not necessarily terminate in the horn, they innervate the skin surrounding the horn, which is also removed during dehorning.

N.FRONTALIS

In the majority of animals dissected the *N.frontalis* was a thin nerve which innervated the lateral frontal region. In these cattle, branches of the *N.frontalis* could not be grossly traced to the horn. However, in one 15-month-old animal the lateral branch of the *N.frontalis* was traced to the rostral aspect of the cut horn base, when dissecting out the nerve for Fluorogold histochemistry. In addition, Fluorogold granules were present in the *N.frontalis* of this animal. Lauwers and De Vos (1966) traced the *N.frontalis* to the horn base in six out of thirty-two animals. As in the animal in the present study, the *N.frontalis* observed by Lauwers and De Vos (1966) branched in a manner similar to that of the *R.zygomaticotemporalis*. In the 15-month-old heifer, anaesthesia of the *R.zygomaticotemporalis* would not have blocked the lateral branch of the *N.frontalis*, the dorsal branch of which ran along the *Linea temporalis* for most of its course.

N.SINUUM FRONTALIUM

In all the animals studied, the *N.sinuum frontaliUM* arose on a common trunk with the *N.frontalis*. This is the most common pattern of origin for the *N.sinuum frontaliUM*, although it may arise from the *R.zygomaticotemporalis* or directly from the *N.ophthalmicus* (Lauwers and De Vos, 1966; Godinho and Getty, 1971).

Lauwers and De Vos (1966) noted that the *N.sinuum frontaliUM* entered the *Sinus frontalis* through a foramen in the medial orbital wall, before ramifying

throughout the *Sinus frontalis* mucosa and bone forming the *Sinus frontalis*. In the present study osmium stained sections of the horn base revealed the presence of canals in the cornual process which contained large nerve fascicles. In addition, several myelinated nerve fascicles were identified in the cornual diverticular lining in animals over two years of age. Endoscopic examination of the *Sinus frontalis* in animals aged between two and four years revealed the presence of thin tortuous nerves in the *Sinus frontalis caudalis*. However, none of the nerves could be traced grossly into the cornual diverticulum. It is most likely that the nerve fascicles seen in the cornual diverticula were branches of the *N.sinuum frontale*, although Merlu (1953) observed branches of the *R.zygomaticotemporalis* reaching the cornual diverticulum mucosa. Lauwers and De Vos (1966) noted the presence of thin nerves entering the cornual diverticula in five out of thirty-two animals. In the present study several nerves similar in size to the branches of the *R.zygomaticotemporalis* were seen in a canal in the cornual process of a 4 year old animal. However, although the nerves in the canals were comparable in size to the nerves in the dermis they were fewer in number. In addition, canals containing nerve fascicles were not a regular feature in the horns studied. Furthermore, the presence of large nerves in the cornual diverticulum appeared to be restricted to animals over 2 years of age. Thus, the *N.sinuum frontale* probably becomes more significant in horn innervation as the animal matures.

Fluorogold granules were not present in the *N.sinuum frontale* of any of the animals dehorned, indicating that in these animals branches from the *N.sinuum frontale* did not reach the cornual diverticulum. Both heifers in the present study were relatively young and had newly formed cornual diverticula. In the two calves studied, the *Sinus frontalis* had not extended into the cornual process. The Fluorogold results are in agreement with the results of osmium tetroxide-staining of nerve fascicles in the cornual diverticular lining. In both

cases large nerve bundles were not found in the cornual diverticula of animals younger than two years.

Nn.CERVICALES

Branches of the *Nn.cervicales* could not be traced to the horn base in any of the animals dissected. In addition no Fluorogold granules were visualized in the *Nn.cervicales* of any of the animals dehorned. Few researchers have noted the innervation of the caudal horn base by the *Nn.cervicales*. However, a few workers have proposed that the *Nn.cervicales* should be blocked in animals in which a large section of skin, caudal to the horn, is removed during dehorning. (Taylor, 1955; Butler, 1967).

GOATS

R.ZYGOMATICOTEMPORALIS

The *R.zygomaticotemporalis* arose as a single or double-stranded fasciculus from the *N.ophthalmicus*. In six of the twenty-four goats studied the two fasciculi did not unite. Getty (1975) observed a double-stranded *R.zygomaticotemporalis* in some goats. Other authors described the *R.zygomaticotemporalis* as a single nerve which ramified close to the horn base and supplied the caudolateral horn base (Vitums, 1954; Thirkell *et al.*, 1990). In the present study, it was found that although the *R.zygomaticotemporalis* predominantly innervated the caudolateral regions, small branches were sometimes distributed to the rostral aspect of the horn. Although variation was seen in the branching pattern of the *R.zygomaticotemporalis*, the variants seen would not cause an anaesthetic failure. In animals 2 to 6-years old, the *R.zygomaticotemporalis* was smaller than the *N.infratrochlearis*, indicating that the *N.infratrochlearis* is the major nerve supplying the horn in adults.

N.INFRATROCHLEARIS

The origin and course of the *N.infratrochlearis* was as described by Sisson and Grossman (1976) and Godinho and Getty (1971). However in the present study great variation in the branching pattern of the *N.infratrochlearis* was seen. The *N.infratrochlearis* divided into between one and three branches under the *M.orbicularis oculi*. The number of branches reaching the horn base varied between individuals. In goats dissected by Thirkell *et al.* (1990) a single-stranded *N.infratrochlearis* emerged from the *M.orbicularis oculi*. Vitums (1954) reported the presence of a double-stranded *N.infratrochlearis*, although only one branch reached the horn base.

The *N.infratrochlearis* is usually blocked on the dorsomedial margin of the orbit (Vitums, 1954; Mitchell, 1966^b; Buttle *et al.*, 1986; Thirkell *et al.*, 1990). However, Skarda (1986) recommended blocking the *N.infratrochlearis* midway between the medial canthus of the eye and the medial aspect of the horn base. The former blocking procedure would have been successful in all the goats studied. The latter procedure, recommended by Skarda (1986) would have been unsuccessful in thirteen of the twenty-four goats in which the *N.infratrochlearis* branched within the *M.orbicularis oculi*.

N.FRONTALIS

No branches from the *N.frontalis* could be traced to the horn base in any of the animals dissected. This is in agreement with findings by Sisson and Grossman (1976) who reported branches of the *N.frontalis* supplying the lateral eyelid and adjacent areas.

N.SINUUM FRONTALIUM

The origin and course of the *N.sinuum frontale* was as reported by Sisson and Grossman (1976). By 6 years of age, the *N.sinuum frontale* had become a substantial nerve with large branches distributed in the cornual diverticulum mucosa. In addition, nerves were found in canals which traversed the cornual process. The nerve fascicles in the cornual diverticulum were larger than the nerves seen in the deep dermis. It is not possible to positively state that these nerves are branches of the *N.sinuum frontale*. However, it is most likely that the *N.sinuum frontale* is the source of these nerves as endoscopy of the *Sinus frontalis* revealed the presence of nerves in the cornual diverticulum which could be traced into the *Sinus frontalis caudalis*.

Nn.CERVICALES

In the present study branches from the *Nn.cervicales* could not be traced to the horn base. This is contrary to findings of Thirkell *et al.* (1990) who, after dissecting goats of various ages, reported that branches of the *N.occipitalis major* and *N.auricularis magnus* reached the caudal horn area in some animals. It is likely that the skin close to the caudal horn base needs to be desensitized by blocking the cervical branches in animals in which the skin is removed during dehorning.

SUMMARY

CATTLE

R.ZYGOMATICOTEMPORALIS

Gross dissections and the use of the retrogradely transported dye, Fluorogold, have shown that the *R.zygomaticotemporalis* is the main nerve supplying the horn base in cattle. In two animals Fluorogold was demonstrated in the *R.zygomaticotemporalis* of the horn base on which Fluorogold had not been applied. This finding suggests that nerves cross over the crown to supply the horn on the opposite side of the head.

In the majority of animals, the *R.zygomaticotemporalis* branched midway between the eye and horn base. However, variations were seen in the branching pattern of this nerve. For example, in one calf the medial fasciculus of the *R.zygomaticotemporalis* took an unusual course, exiting the orbit through a foramen caudodorsal to the orbital rim. In two other animals a *R.cornualis* left the *R.zygomaticotemporalis* as it emerged from the orbit.

N.INFRATROCHLEARIS

R.cornuales from the *N.infratrochlearis* could be traced to the horn base in two out of twenty-five animals dissected. Despite this, Fluorogold granules were present in the *Nn.infratrochlearis* of all four animals used in the Fluorogold study. This finding suggests that the *N.infratrochlearis* innervates the skin close to the horn base.

N.FRONTALIS

The *N.frontalis* supplied the horn base of one animal studied. This was confirmed by the presence of Fluorogold granules in the *N.frontalis* of this animal.

N.SINUUM FRONTALIUM

The *N.sinuum frontale* did not appear to innervate the horn base of animals below 2 years of age. Nerve bundles were demonstrated in the cornual diverticula of animals over 2 years of age.

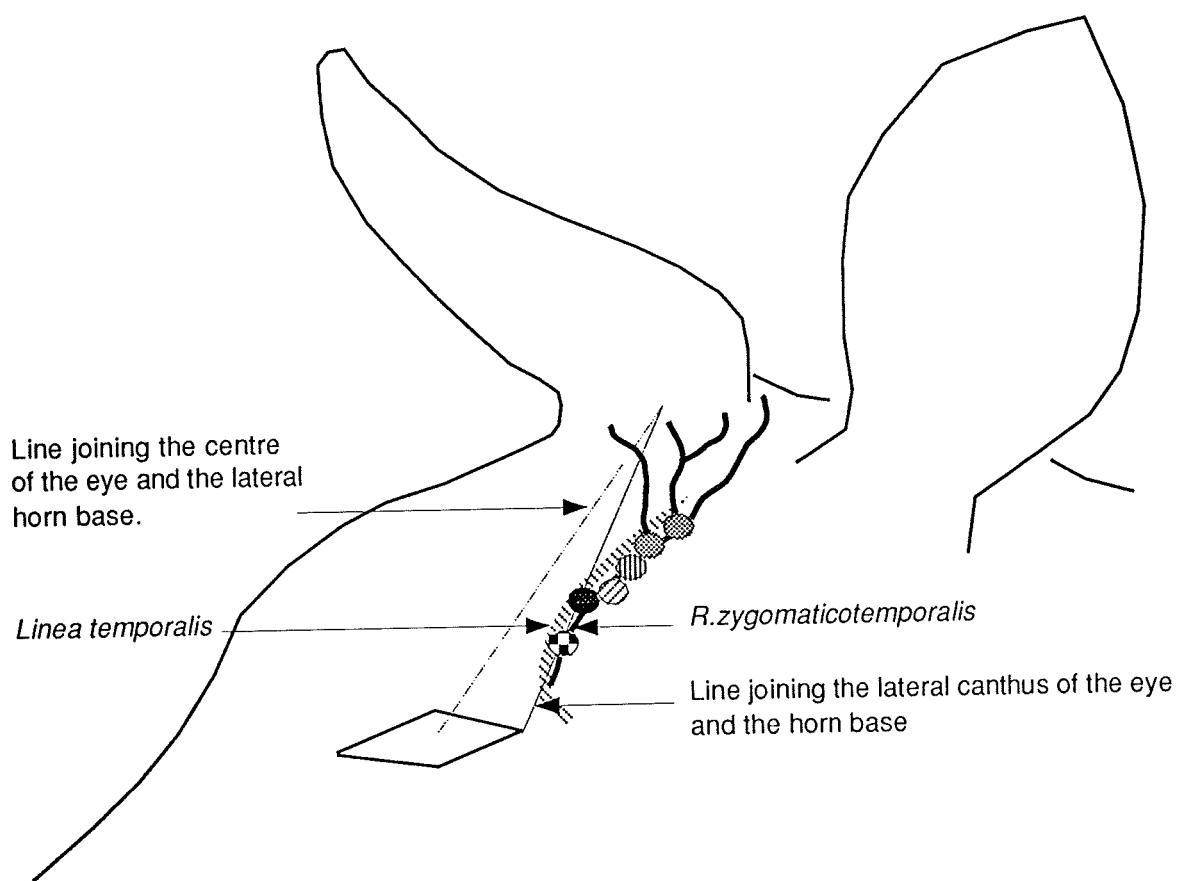
Nn.CERVICALES

Branches from the *Nn.cervicales* could not be grossly traced to the horn base in any of the animals dissected. In addition, no Fluorogold granules were found in the *Nn.cervicales* of the four animals studied.

GOATS

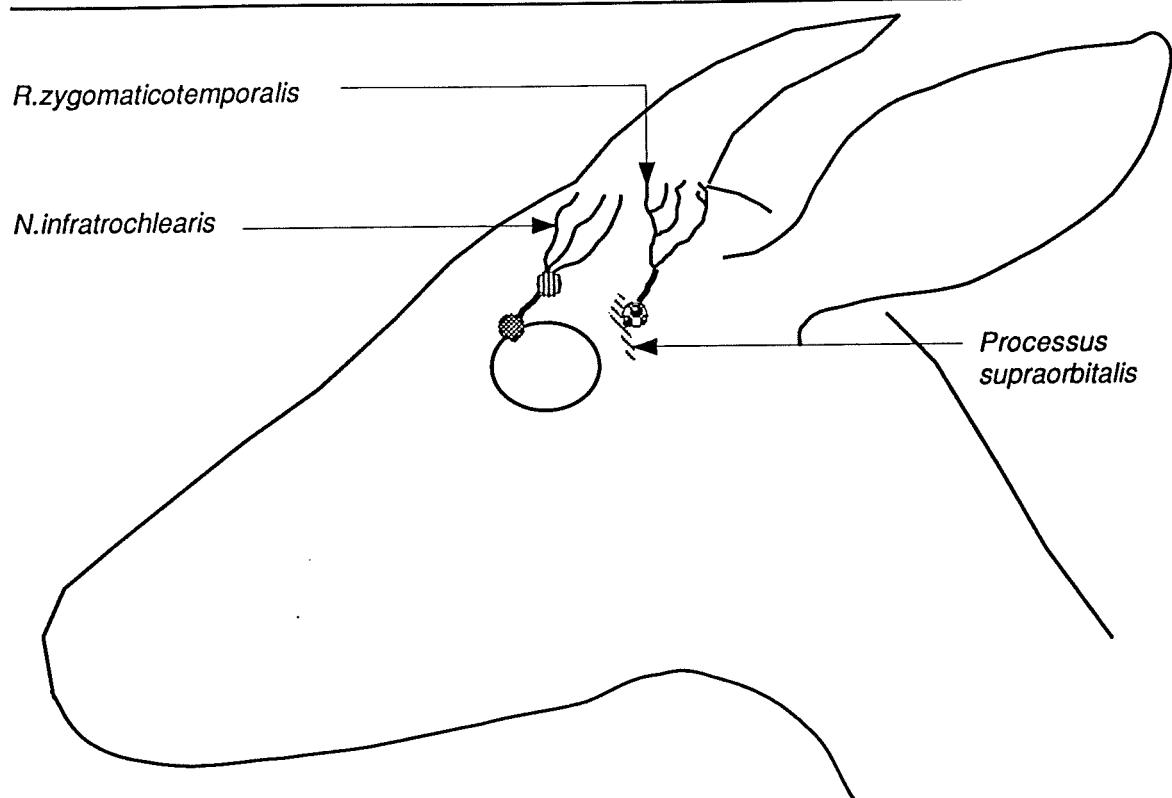
The *R.zygomaticotemporalis* and *N.infratrocchlearis* were the two nerves supplying the horn base in all the goats dissected. However, branches of the *N.sinuum frontale* were demonstrated in the cornual diverticula of animals over 4 years of age. In animals over 2 years the *R.zygomaticotemporalis* appeared thinner and less extensive than the *N.infratrocchlearis*. Various branching patterns of the *R.zygomaticotemporalis* and *N.infratrocchlearis* were observed.

No branches from either the *N.frontalis* or *Nn.cervicales* could be traced to the horn base in any of the animals studied.

KEY

- Emmerson (1933)
- Browne (1938)
- Tazewell and Greenough (1950); Butler (1967)
- Wheat (1950); Merlu (1953)
- Mitchell (1966^a); Horney (1966)

Figure 2.1. Schematic diagram showing the blocking sites for the *R.zygomaticotemporalis* in cattle.



KEY

- Ⓐ Blocking site for the *R.zygomaticotemporalis* (Vitums, 1954; Skarda, 1986)
- Ⓑ Blocking site for the *N.infratrochlearis* (Vitums, 1954)
- Ⓒ Blocking site for the *N.infratrochlearis* (Skarda, 1986)

Figure 2.2. Schematic diagram showing the blocking sites for the *R.zygomaticotemporalis* and *N.infratrochlearis* in goats.

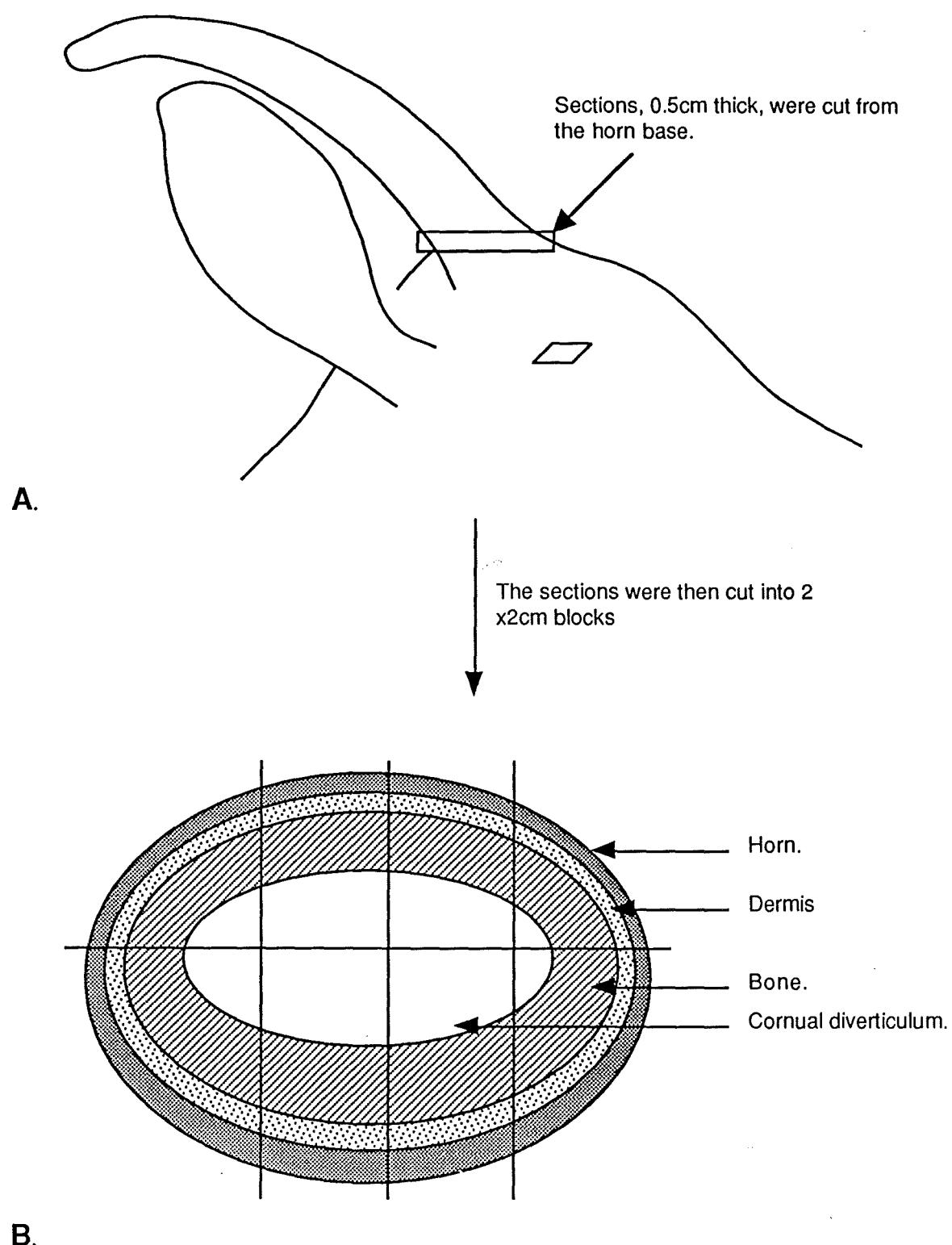


Figure 2.3. A. Diagram showing the location from which sections were removed from the horn base.

B. Horn base sections were cut into 2 x 2 cm blocks.



Figure 2.4. Photograph of the 5-week-old calf.

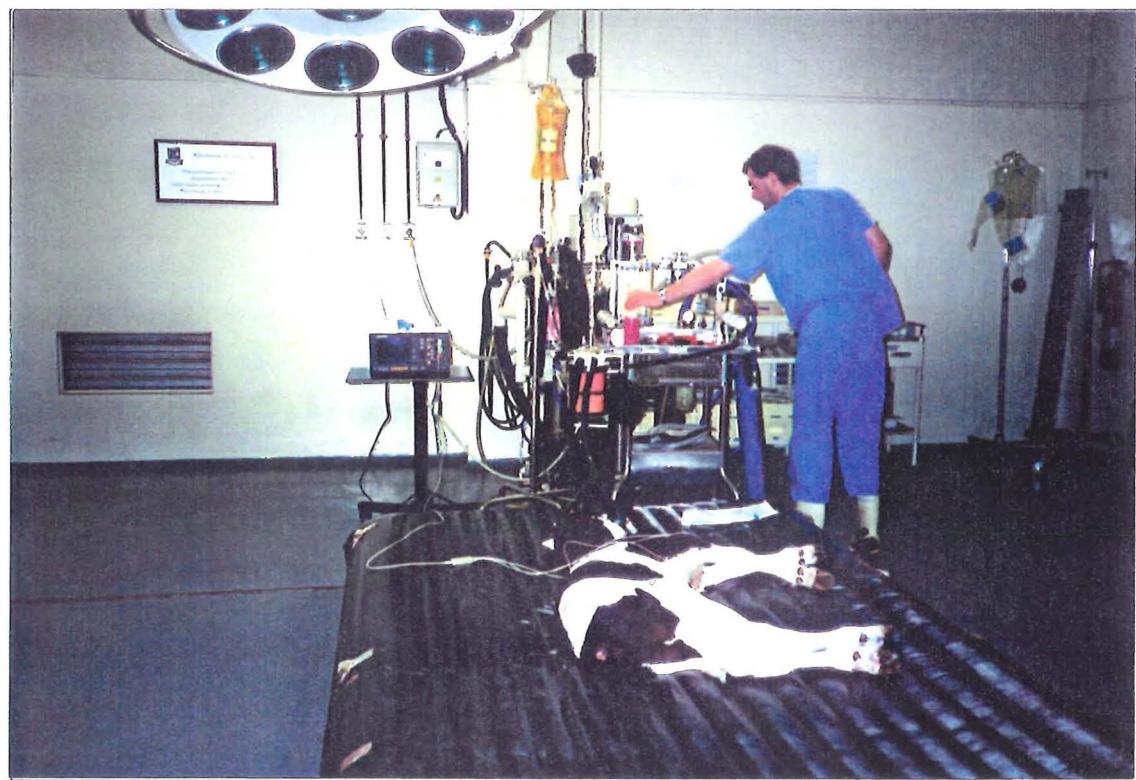


Figure 2.5 The disbudding of the calves and the application of Fluorogold were performed with the calves under general anaesthesia.



Figure 2.6 The area surrounding the horn buds was clipped and surgically prepared.

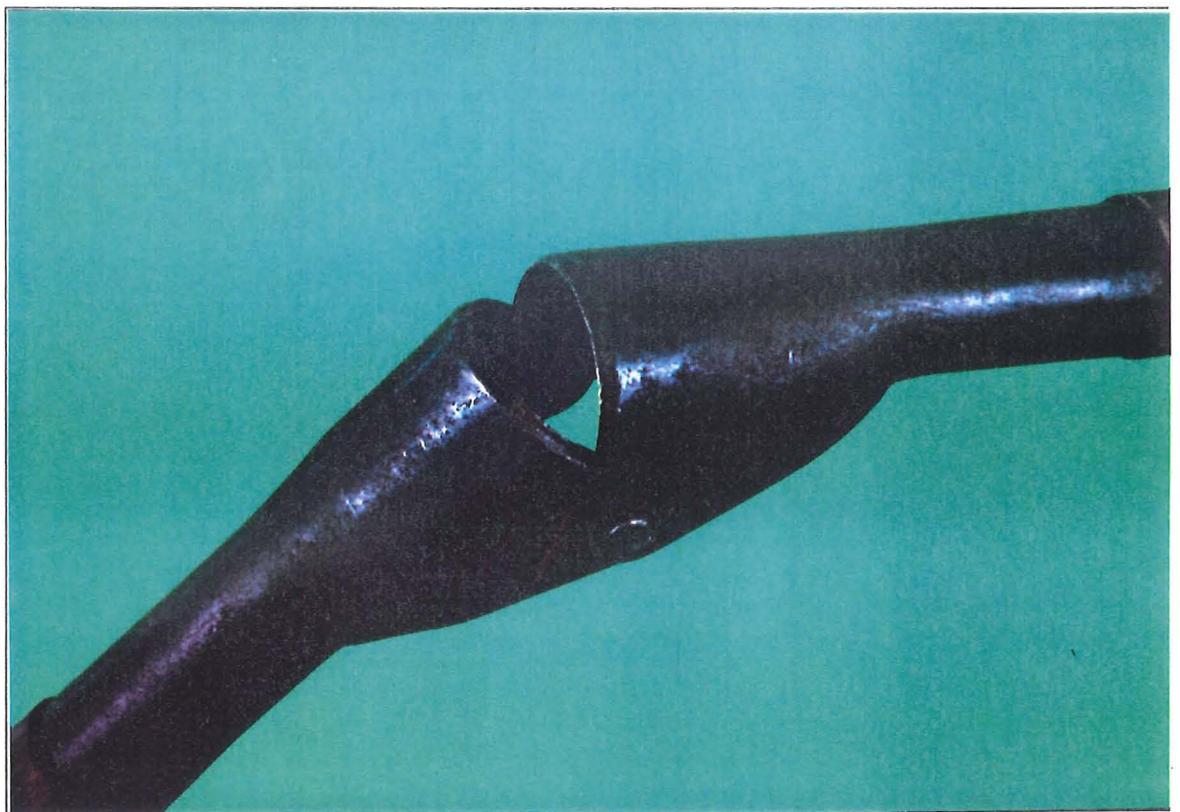


Figure 2.7. Small disbudding shears with curved sharp edges were used to disbud the calves.



A



B

Figure 2.8. A. The sharp edges of the shears were applied to the base of the horn bud.

B. The developing cornual process and all of the developing horn were removed .

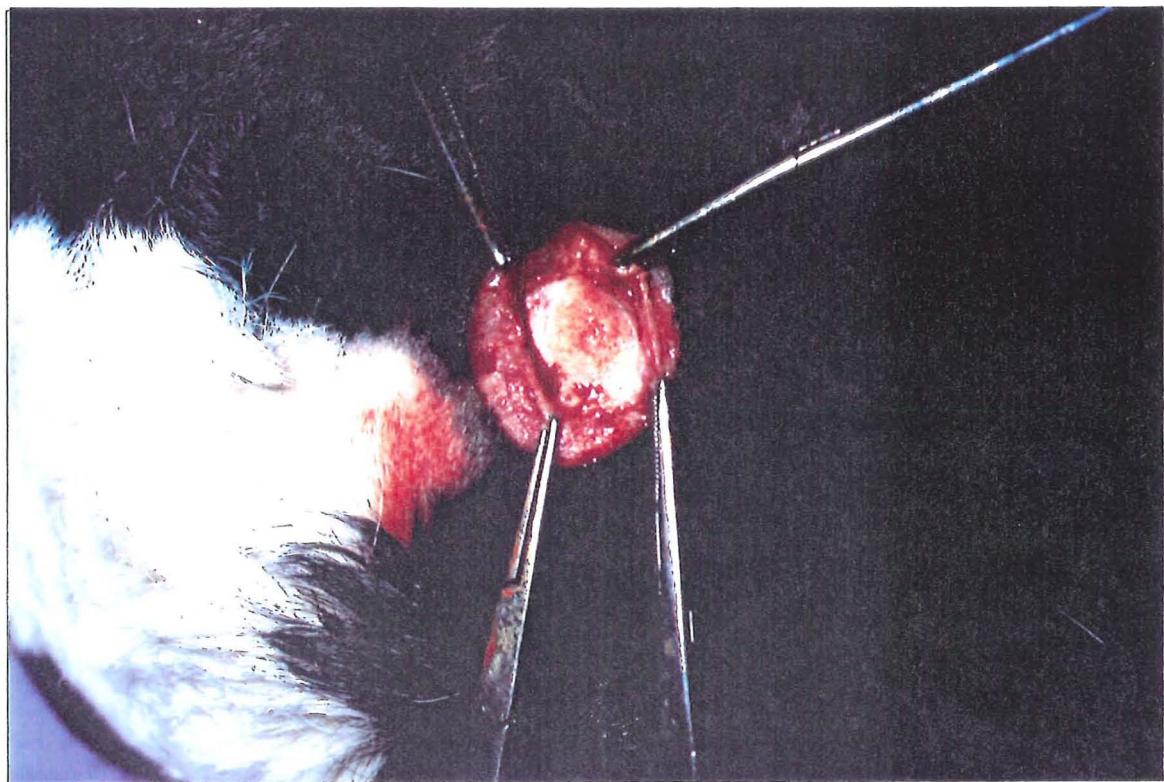


Figure 2.9. Haemorrhage was controlled using haemostatic forceps.

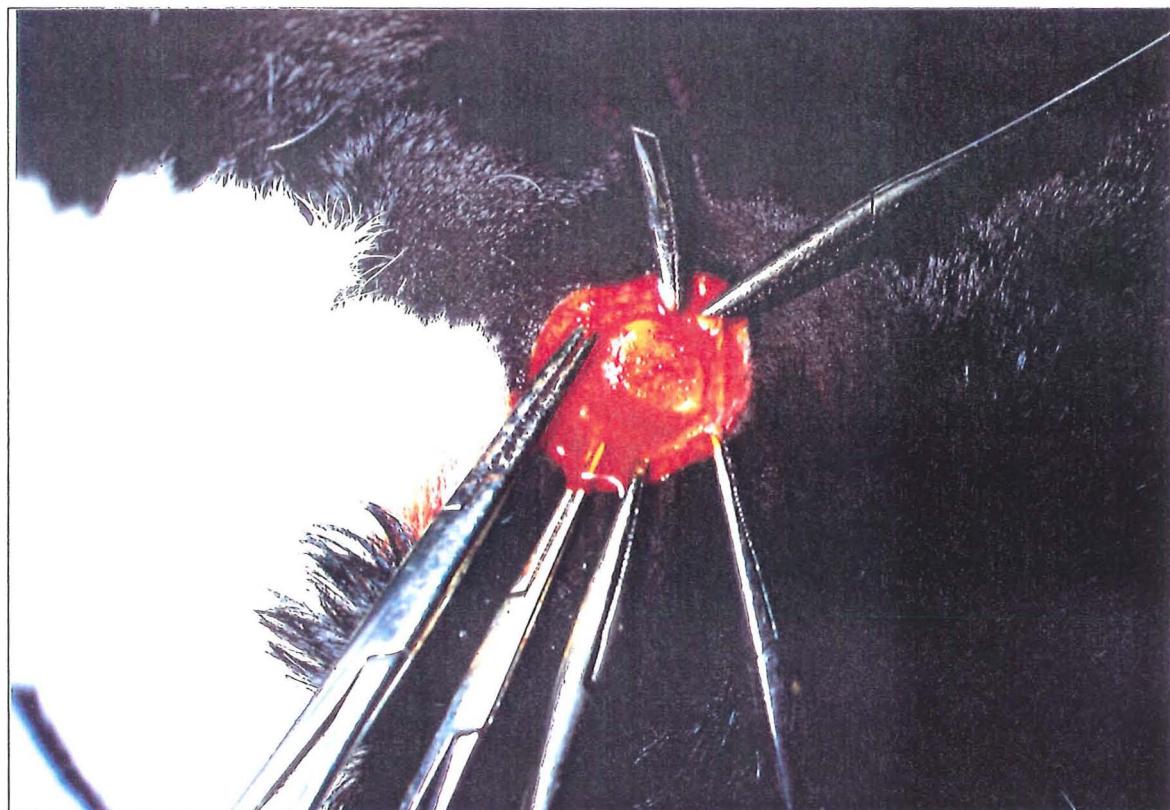


Figure 2.10 Fluorogold dissolved in sterile distilled water was applied to the wound and left undisturbed for 30 minutes.

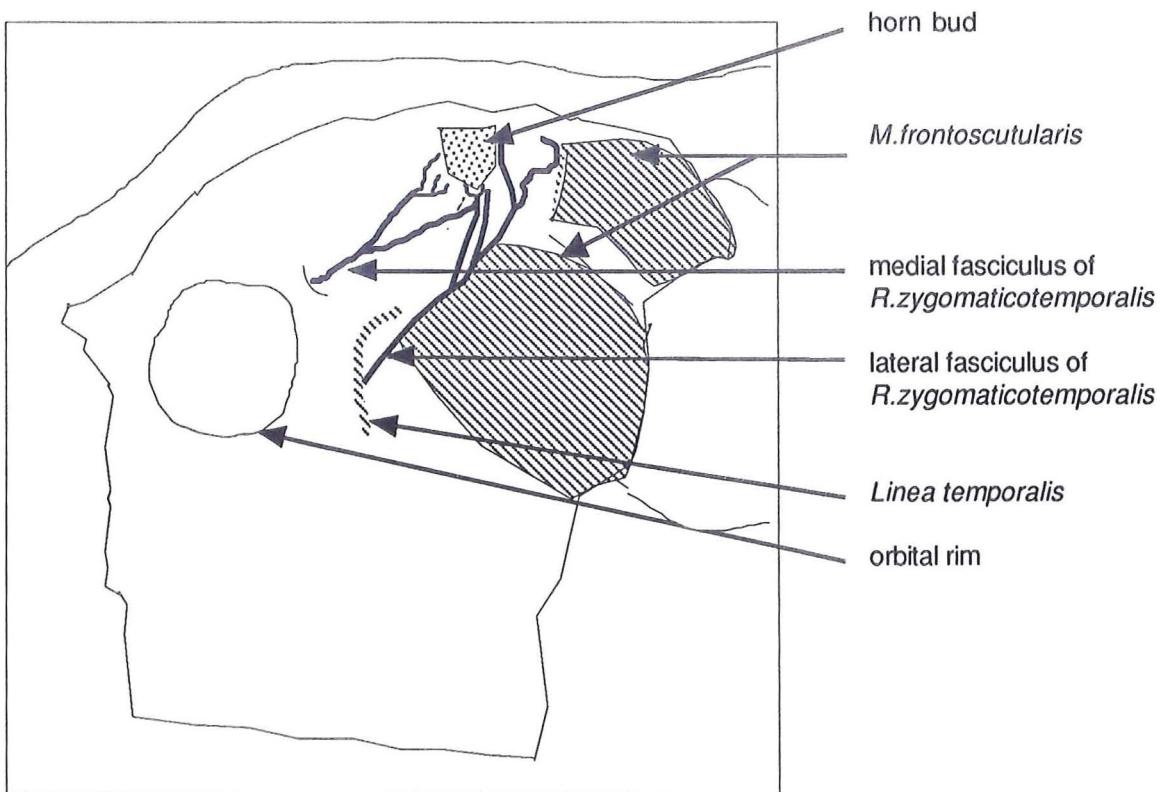
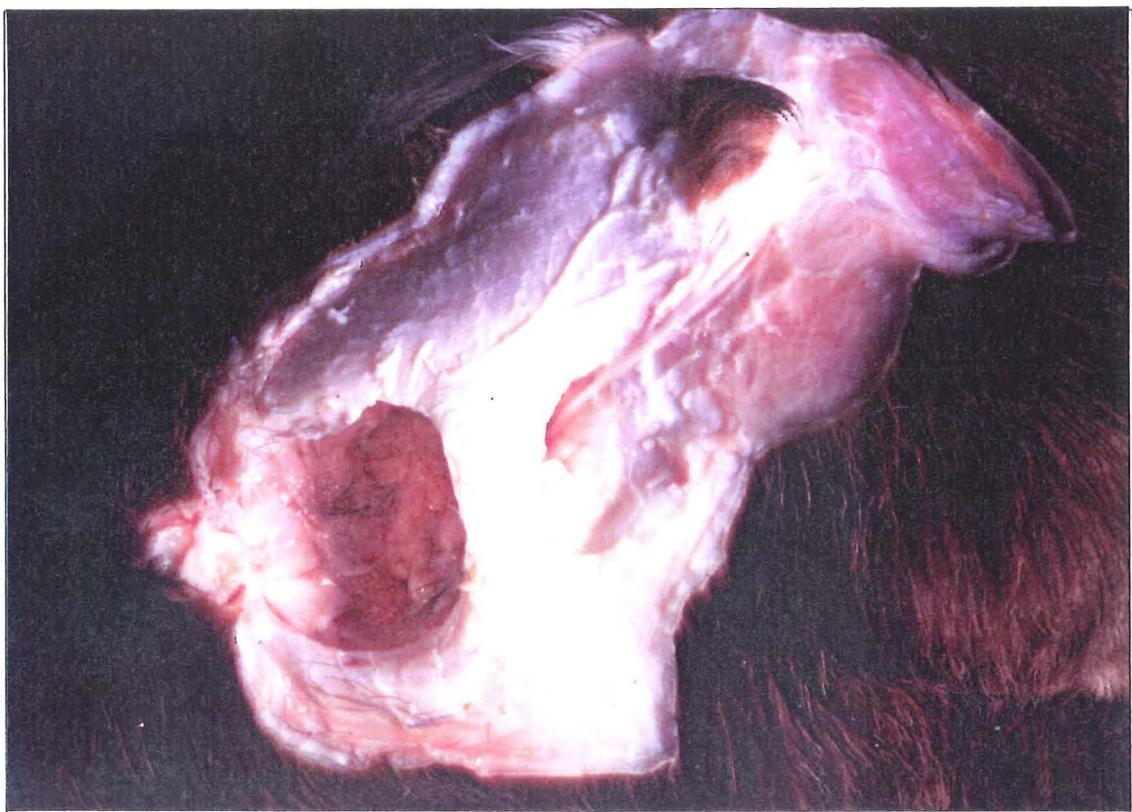


Figure 2.11. Dissection of the nerve supply to the horn of a 3-week-old calf. In this animal the medial fasciculus of the *R.zygomaticotemporalis* takes an unusual course through a foramen in the *Os frontale*.

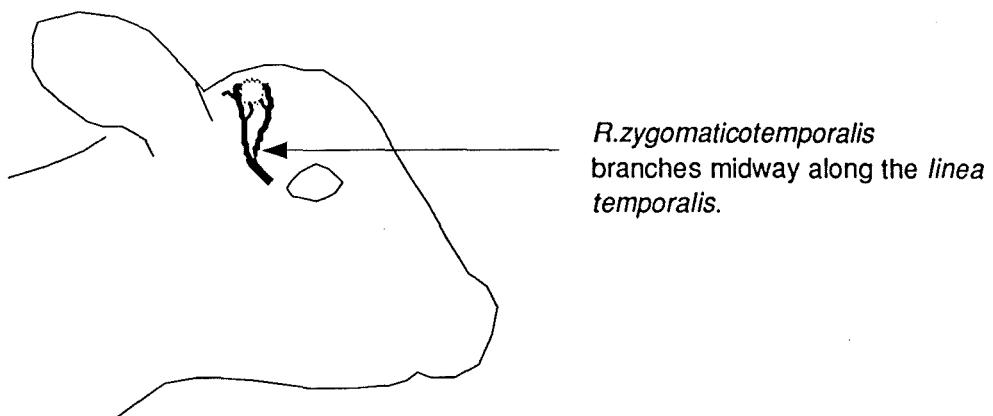


FIGURE 2.12.A. The *R.zygomaticotemporalis* divides into two branches midway between the eye and horn. This was the most common branching pattern and was seen in the majority of calves dissected.

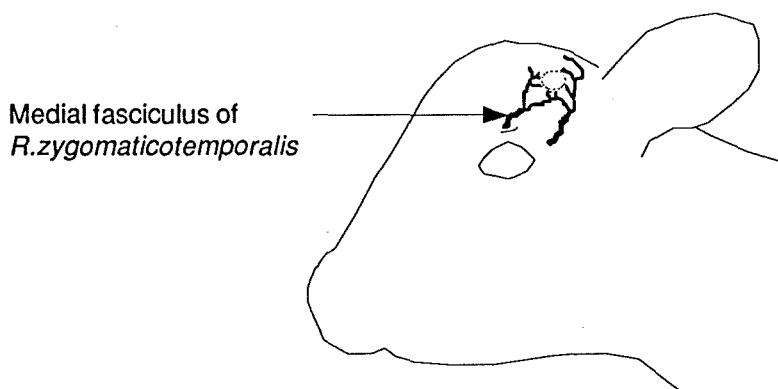


Figure 2.12. B. The medial fasciculus of the *R.zygomaticotemporalis* exits the orbit through a foramen in the *Os frontale*. Branches from this nerve innervate the rostromedial and rostralateral aspects of the horn. This branching pattern was seen in one of the ten calves dissected

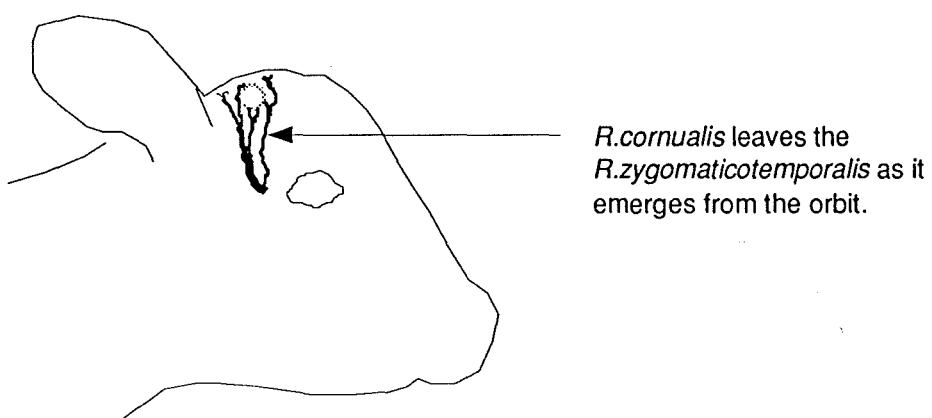


Figure 2.12 C A *R.cornualis* leaves the *R.zygomaticotemporalis* as it emerges from the orbit. The *R.cornualis* innervates the rostromedial aspect of the horn. This branching pattern was seen in a 4-week-old calf.

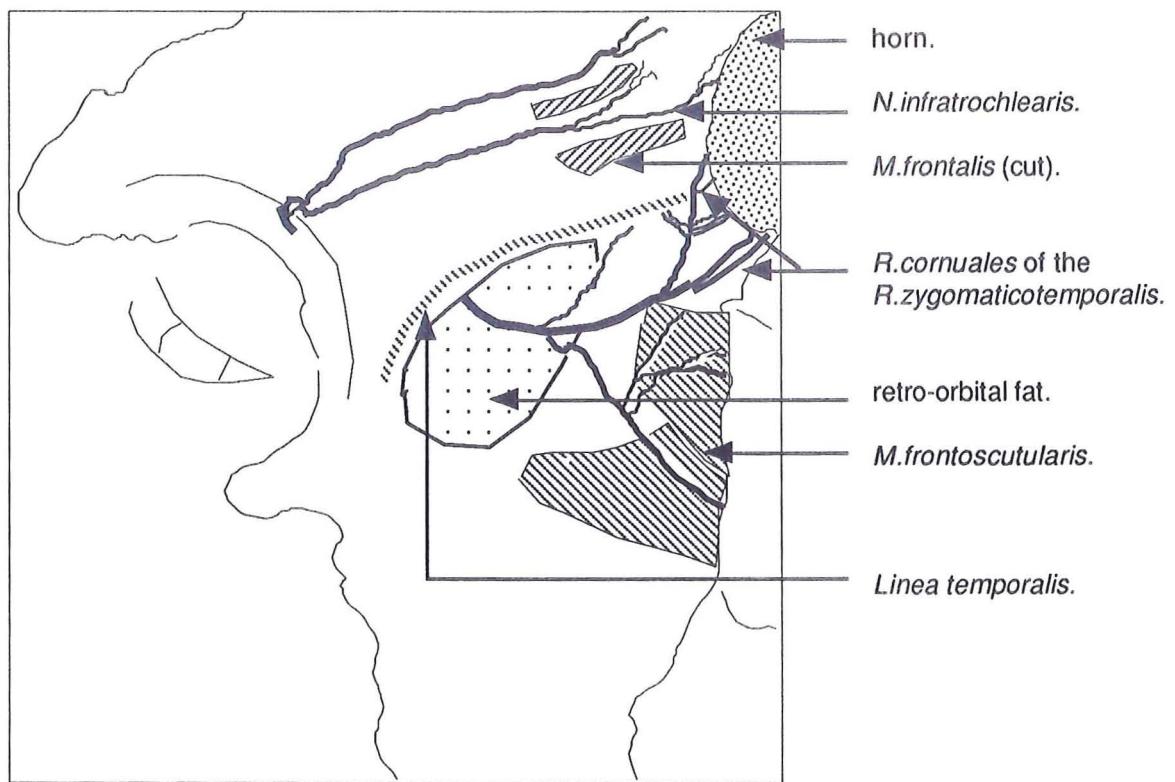
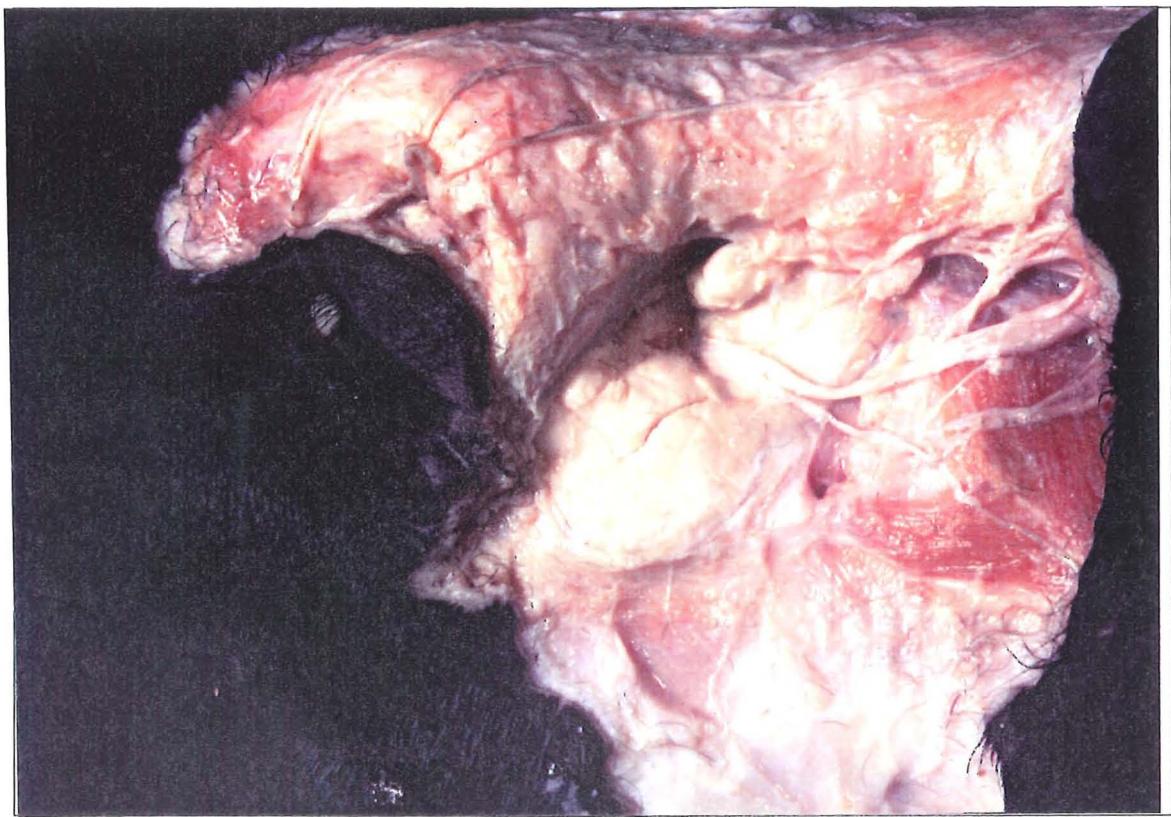


Figure 2.13 Dissection of the nerves supplying the horn in a 3-year-old bovine male. The *R.zygomaticotemporalis* supplies the rostral, caudal and lateral aspects of the horn, whilst a branch of the *N.infratrochlearis* innervates the medial region of the horn.

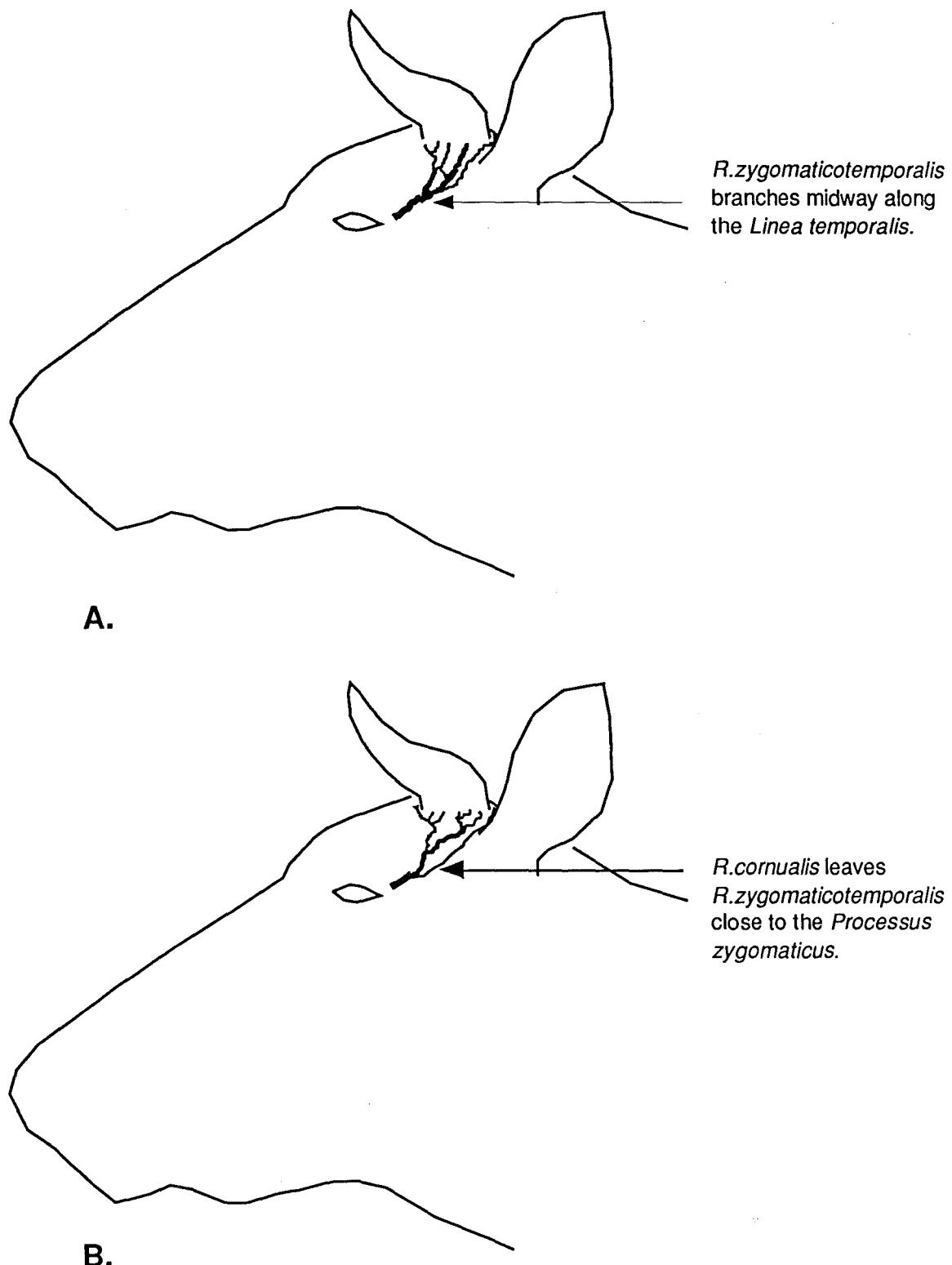


Figure 2.14 A. In the majority of the adult cattle dissected the *R.zygomaticotemporalis* divided midway between the eye and horn.
B. In one of the 3-year-old animals a *R.cornualis* left the *R.zygomaticotemporalis* as it emerged from the orbit. The *R.cornualis* innervated the caudal and medial regions of the horn.

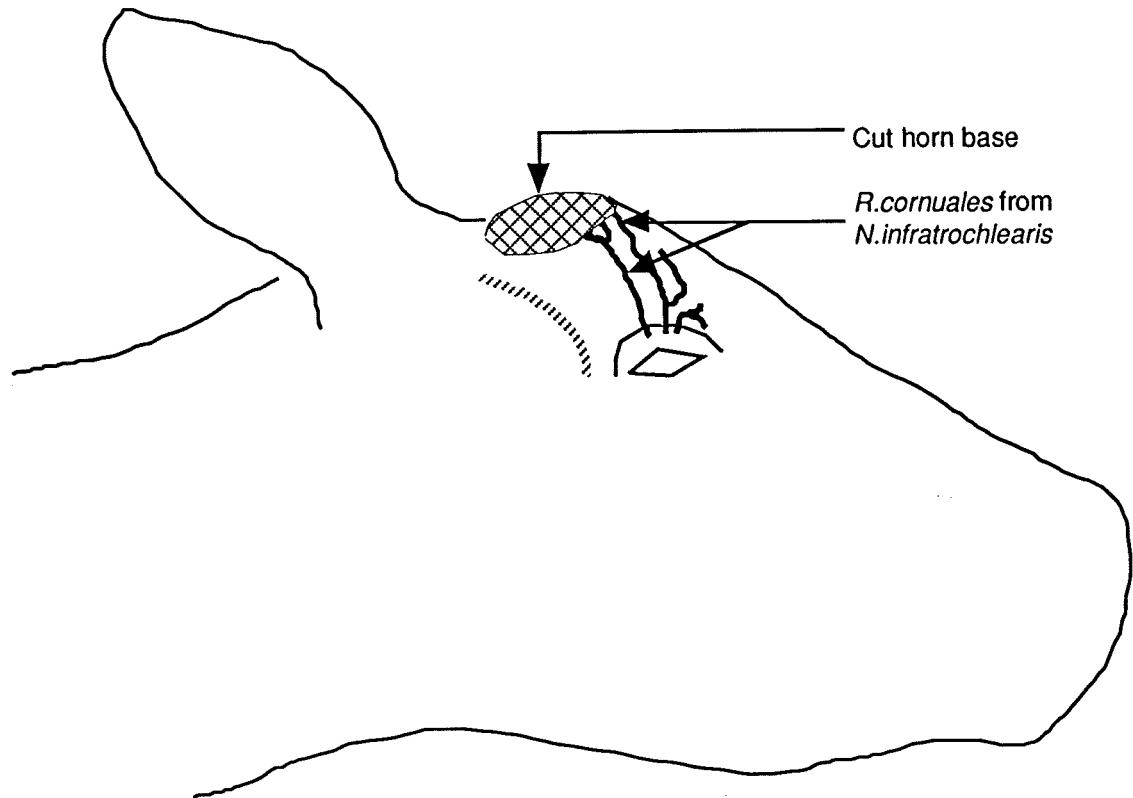


Figure 2.15 15-month-old heifer. Two *R.cornuale* from the *N.infratrocchlearis* innervate the rostral and medial regions of the horn base.

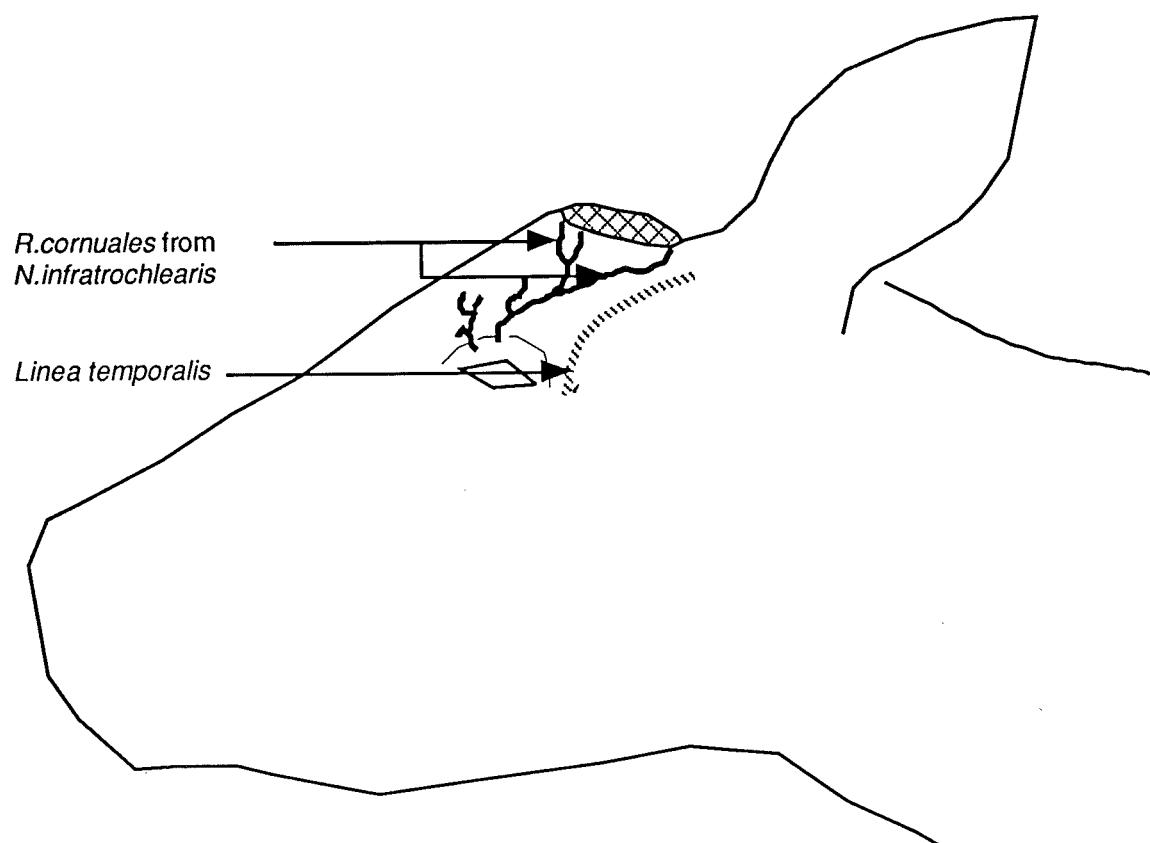


Figure 2.16 15-month-old heifer (same animal as in Figure 2.15). *R.cornuale* from the *N.infratrocchlearis* supply lateral and rostral regions of the horn base on the left side.

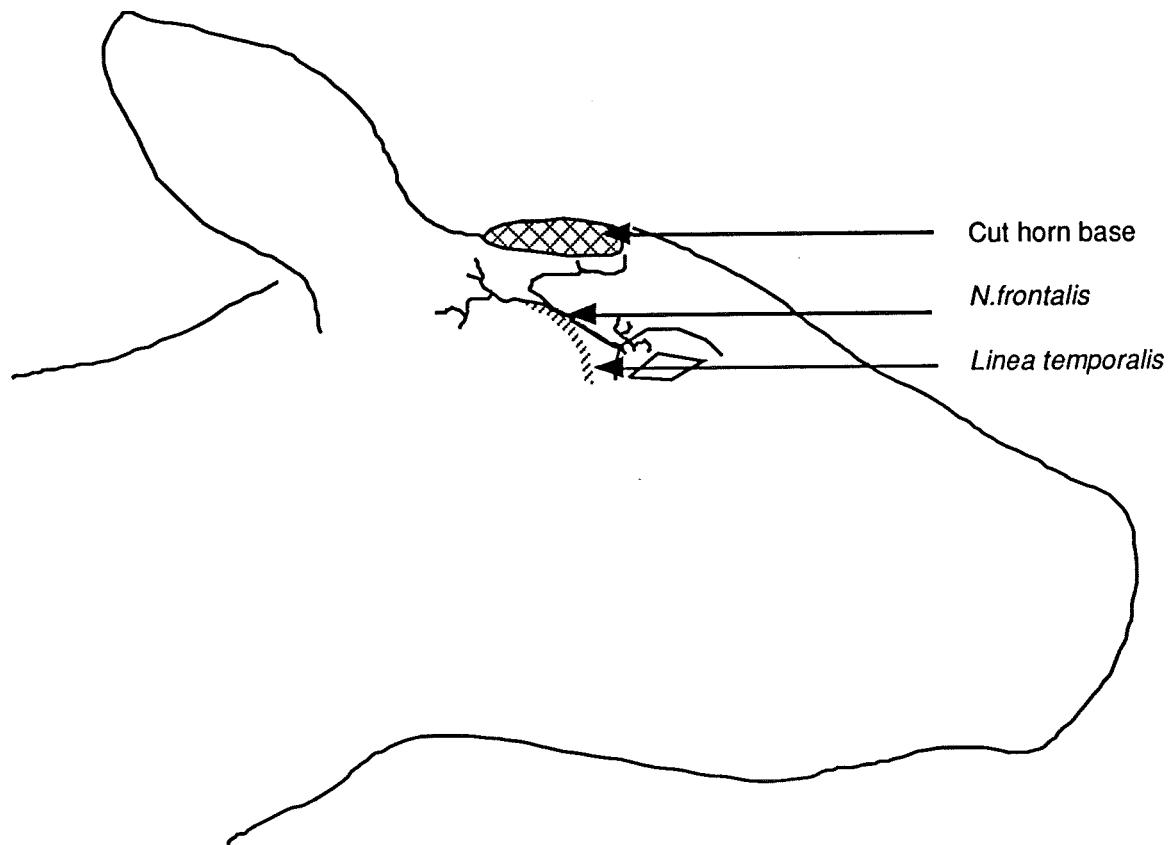


Figure 2.17 15-month-old heifer. Diagram showing an unusual course of a branch of the *N. frontalis*.

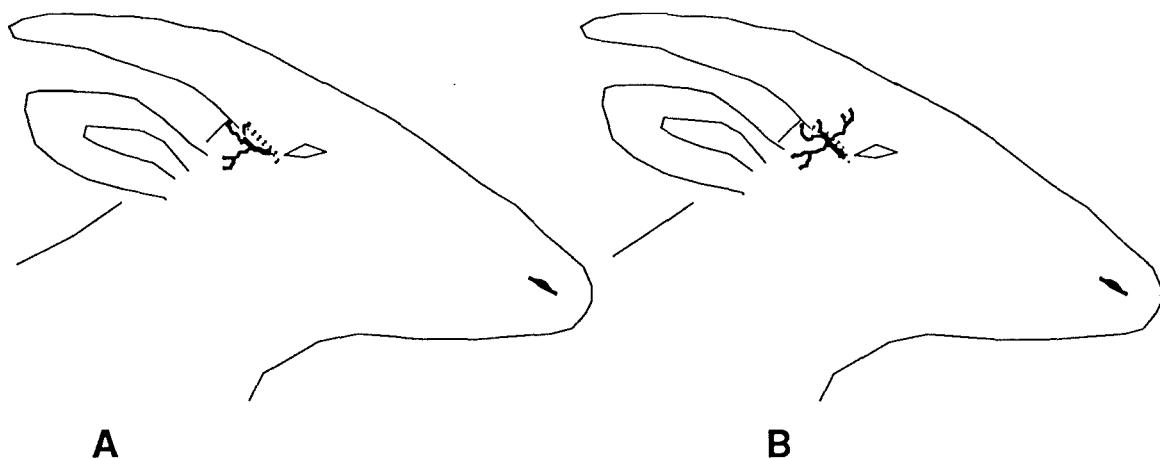
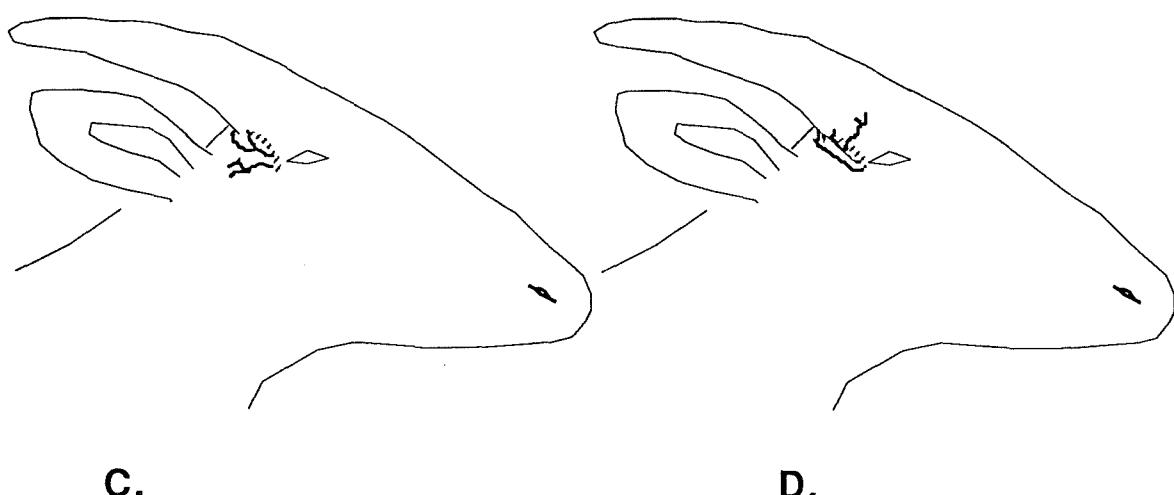


Figure 2.18 A. *R.zygomaticotemporalis* emerges as a single nerve from the orbit. The nerve divides into branches which innervate the caudolateral horn base. This pattern was seen in nine of the twenty-four goats dissected.

B. *R.zygomaticotemporalis* emerges from the orbit as a single nerve which then divides into three branches; two innervate the caudolateral horn base, whilst the third branch innervates the rostral horn area. This branching pattern was found in nine of the twenty-four goats dissected.



C. *R.zygomaticotemporalis* emerges as a double-stranded nerve. The dorsal fasciculus innervates the caudolateral aspects of the horn. This pattern was observed in four goats.

D. A double-stranded *R.zygomaticotemporalis* exits the orbit. Branches are distributed to the caudolateral and rostral regions of the horn base. This branching pattern was seen in two goats.

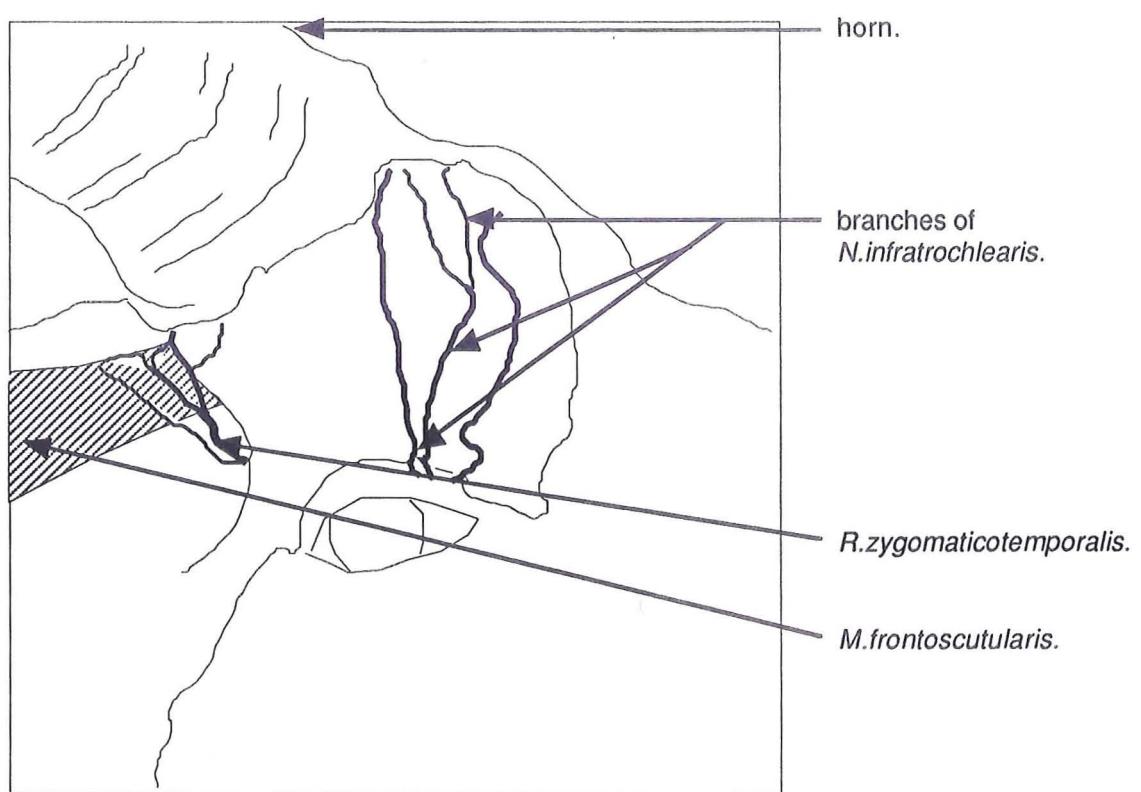
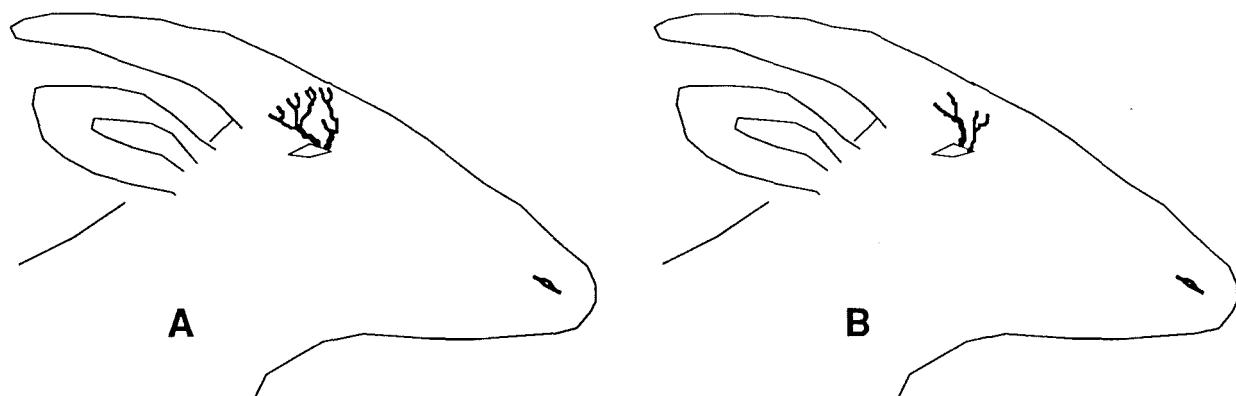
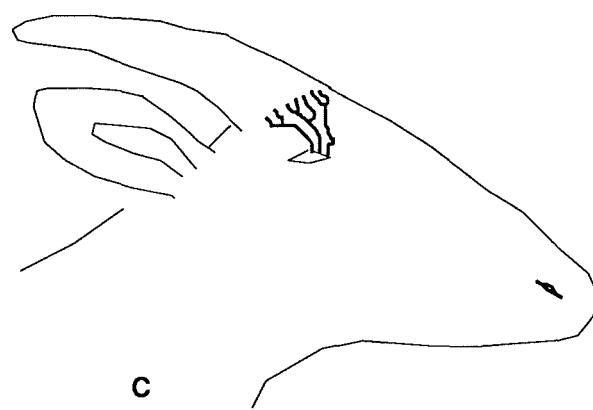


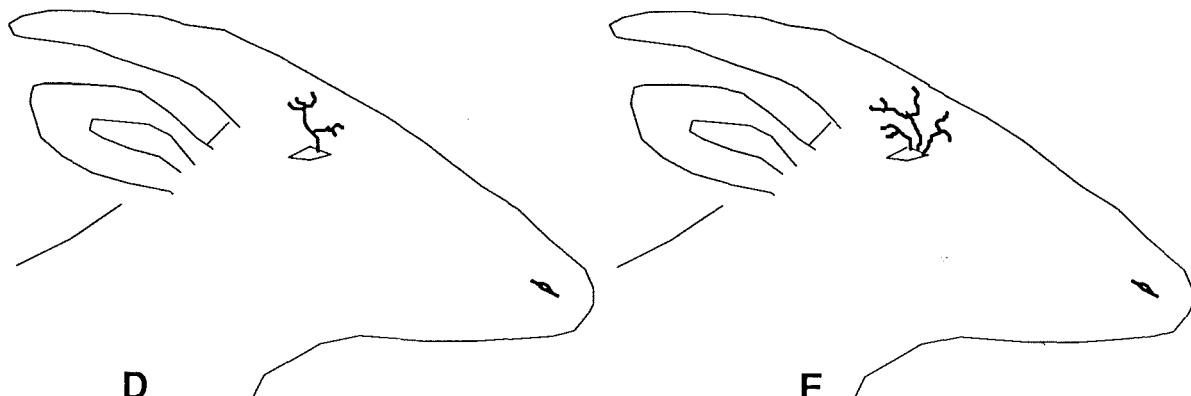
Figure 2.19 Dissection of the nerves supplying the horn in a 6-year-old goat. The *N.infratrochlearis* branches extensively to supply the rostral aspect of the horn. The *R.zygomaticotemporalis* innervates the lateral horn base.



- Figure 2.20.** A. Two branches of the *N.infratrochlearis* emerge from the orbit. Both branches innervate the horn base. This branching pattern was observed in eight of the goats dissected.
- B. Two branches of the *N.infratrochlearis* emerge from the orbit, but only one of these branches reaches the horn base. This pattern was found in six of the goats dissected.



- C. Three branches of the *N.infratrochlearis* emerge from the orbit; all three branches innervate the horn base. This was found in five of the goats dissected.



- D. The *N.infratrochlearis* emerges as a single nerve which branches close to the horn base. This pattern occurred in four of the goats dissected.
- E. Three branches of the *N.infratrochlearis* emerge from the orbit, but only one branch reaches the horn base. This pattern was seen in one animal.



Figure 2.21. An osmium-stained nerve bundle (arrow) in the cornual diverticular lining of a 2-year-old bull. Osmium stain. X 375.

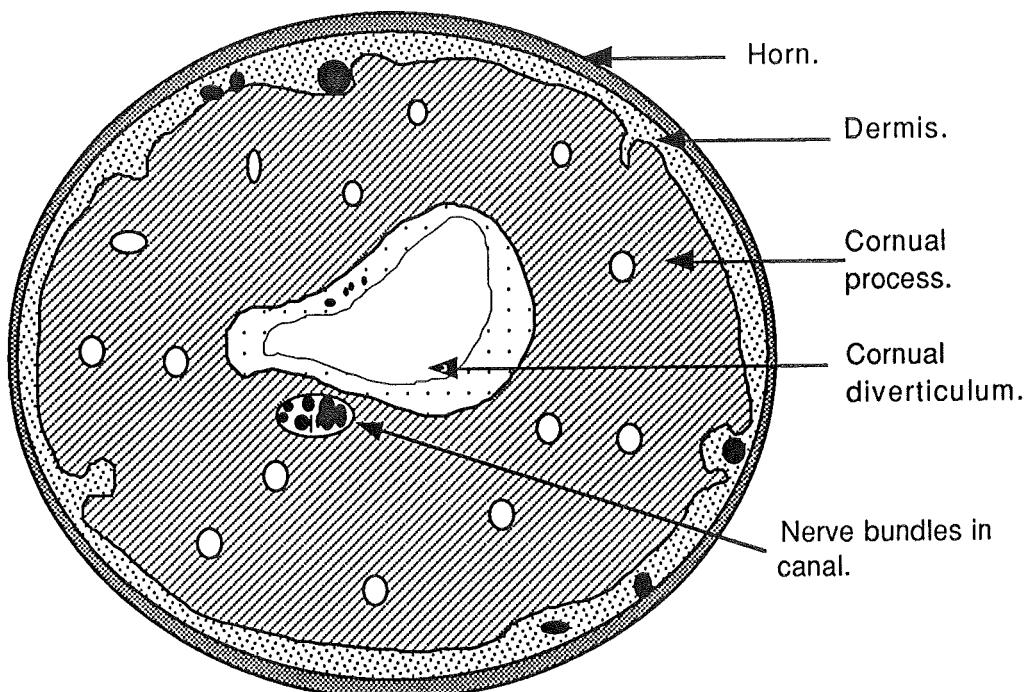


Figure 2.22. Schematic illustration of a cross section through the horn of a 4-year-old bull. In this animal a canal containing several nerve fascicles was observed in the cornual process.

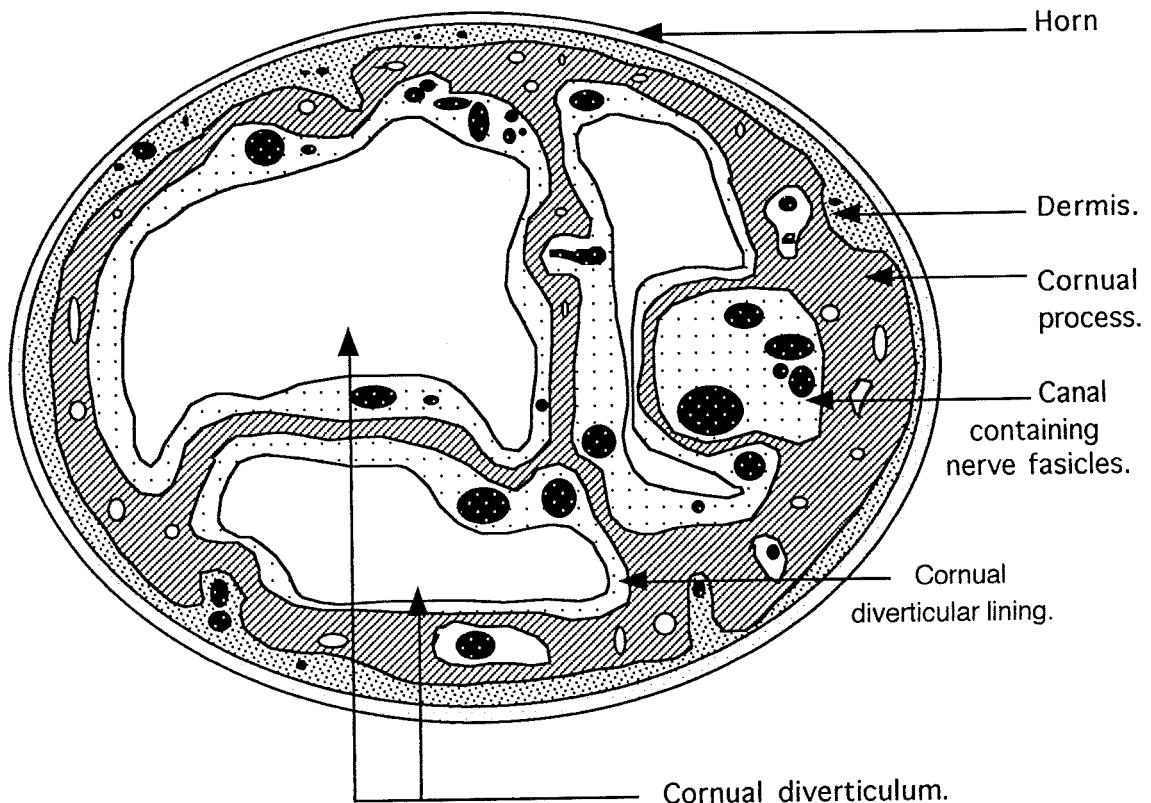


Figure 2.23. Cross section through the horn base of a 6-year-old goat. Numerous nerve fascicles are scattered throughout the cornual diverticular lining. In addition, a canal containing several nerves fascicles is present in the compact bone of the cornual process.

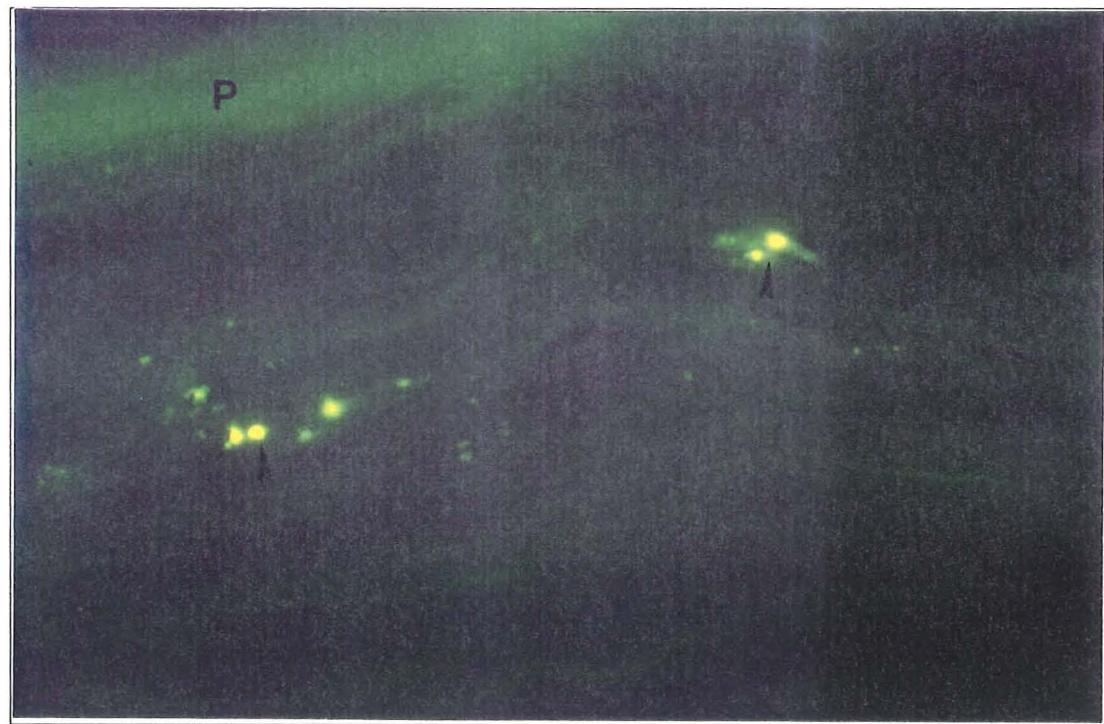
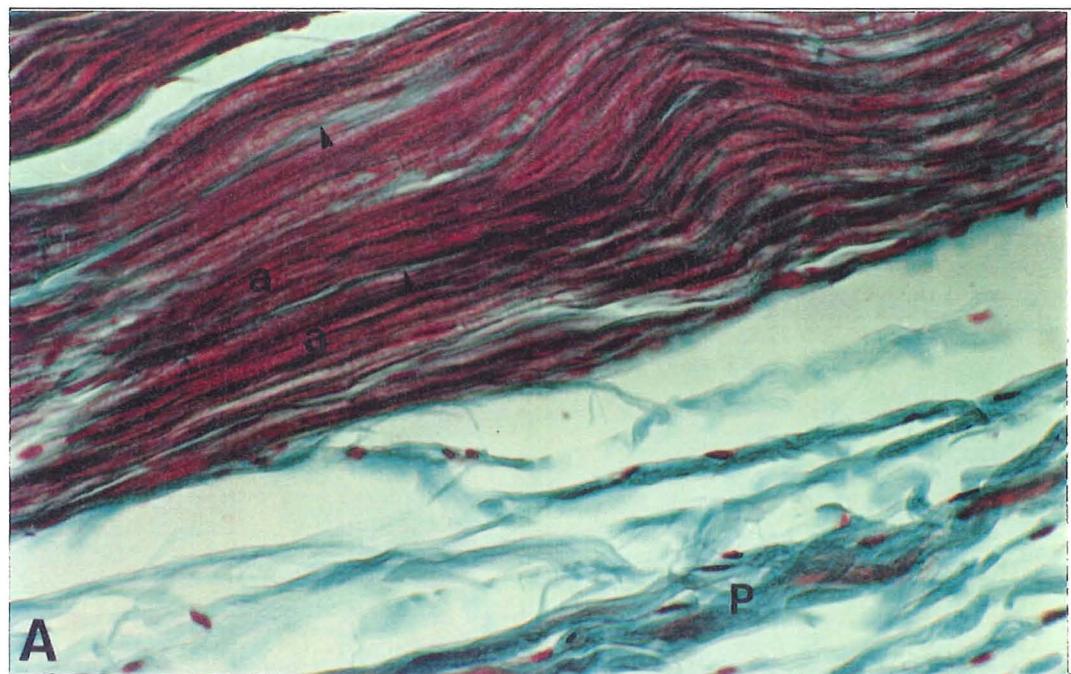


Figure 2.24. A. Longitudinal section of a nerve fascicle composed of axons (a) which are separated by endoneurium (arrowheads). The bundle of axons is surrounded by a perineural sheath (p). Masson's trichrome stain. X 750.

B. Fluorogold granules (arrowheads) in the *R.zygomaticotemporalis* of a 15-month-old heifer. The endoneurium and perineurium (p) fluoresce green, whilst the axons are non-fluorescent. X 750.

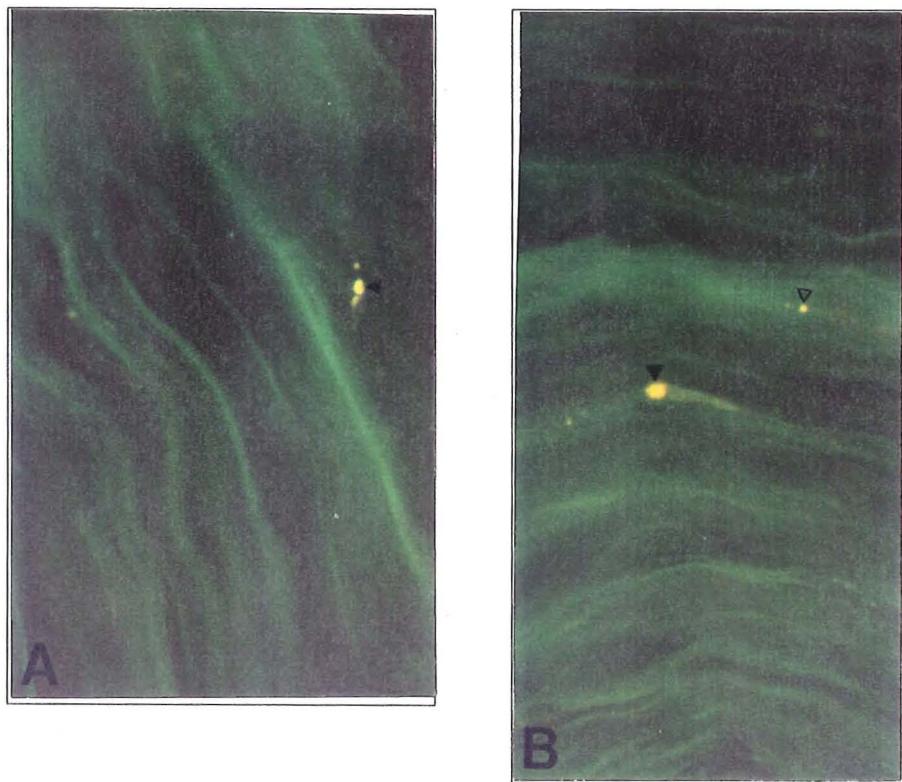


Figure 2.25 A. 15-month-old heifer. Fluorogold granules (arrowhead) in the *R.zygomaticotemporalis* of the control horn base. X 750.

B. 15-month-old heifer. Fluorogold granules in the *N.frontalis*. Small granules (clear arrowhead), as well as clumps of granules (black arrowhead) are present. X 750.

CHAPTER 3

THE DEVELOPMENT OF HORN INNERVATION: AN IMMUNOHISTOCHEMICAL AND ULTRASTRUCTURAL STUDY.

INTRODUCTION

Current legislation in Britain states that disbudding of calves and kids must be performed under local anaesthesia unless chemical cauterisation is done during the first week of life (Crofts, 1987). However, European Union law allows the disbudding of calves less than 4 weeks old without the use of local anaesthesia (Morisse *et al.*, 1995). Although several studies (Boandl *et al.*, 1989; Wohlt, 1994; Morisse *et al.*, 1995; Hemsworth *et al.*, 1995; Petrie *et al.*, 1996) report the stress caused by disbudding, it is generally believed that the pain inflicted on the calf or kid at this stage is minimal as the horn bud is poorly developed. However, it has not been determined whether the innervation of the horn bud is poorly developed in calves and kids less than 4 weeks old. The aim of this study was therefore to trace the development of sensory nerve endings in pre-natal and post-natal calves and kids. A systematic study of the sensory innervation in post-natal calves and kids provides information on the sensory receptors present in the post-natal animal, as well as the changes that occur in the innervation as the horn bud changes from an hairy to a non-hairy integument.

LITERATURE REVIEW

Several types of sensory nerve endings have been identified both in the skin and in its derivatives. The sensory receptors so far identified are: corpuscular receptors (Meissner's, Pacinian, Ruffini), perifollicular terminals, free nerve endings, Merkel cells and intraepidermal nerves. Ruffini corpuscles and perifollicular terminals are found in association with hair follicles. A review of the structural morphology and development of these sensory receptors is given below. A summary of the sensory endings identified in the skin and the techniques used to demonstrate the endings is shown in Table 3.1.

CORPUSCULAR RECEPTORS

Corpuscular receptors are composed of an axon terminal surrounded by Schwann cells or lamellar cells, the whole structure usually being enclosed in a capsule. Meissner's, Pacinian and Ruffini corpuscles have been recognized in skin. Simplified versions of Pacinian and Meissner's corpuscles have also been identified and are termed bulbous and coiled corpuscles respectively.

MEISSNER'S CORPUSCLES

Meissner's corpuscles are found in large numbers in non-hairy skin where they function as rapidly adapting mechanoreceptors responding to touch (Guyton, 1991). Meissner's corpuscles are located in the papillary layer of the dermis, where they are in close contact with the epidermal cells at the tip of the dermal papillae (Ridley, 1969; Halata and Munger, 1983). In the human hand half of the dermal papillae contain Meissner's corpuscles (Miller *et al.*, 1958). The corpuscles occur either singly or in groups of up to eight (Miller *et al.*, 1958). The cellular component of Meissner's corpuscle is made up of lamellar cells which are stacked horizontally, parallel to the epidermal surface (Cauna and Ross, 1960). Immunohistochemical studies have shown that lamellar cells are immunoreactive for the glial specific protein S-100, thus suggesting that they are modified Schwann cells (Iwanaga *et al.*, 1982; Bjorklund *et al.*, 1986; Vega *et al.*, 1996). One end of the lamellar cell contains a flattened nucleus whilst the other end is drawn out into long cytoplasmic processes, between which lie flattened axons (Halata and Munger, 1983; Ramieri *et al.*, 1992). Numerous lamellar profiles are present due to the branched cytoplasmic processes extending to one end of the corpuscle and then doubling back towards the nucleus (Chouchkov, 1973). The lamellar cell cytoplasm contains numerous mitochondria, clumps of ribosomes and well-developed rough endoplasmic

reticulum. Small pinocytotic vesicles are found in the perinuclear area, or attached to the plasma membrane adjacent to axonal endings (Cauna and Ross, 1960). The cytoplasmic processes contain relatively few organelles, a few mitochondria, free ribosomes and cytoplasmic fibrils being the only organelles present in the processes (Chouchkov, 1973). The intercellular spaces between lamellar cells contain randomly orientated collagen fibres, embedded in an amorphous material (Cauna and Ross, 1960). In some areas the intercellular connective tissue and basal lamina, which usually enclose the cells, are absent, thus allowing adjacent lamellar cells to form desmosome-like connections (Chouchkov, 1973).

Each corpuscle is supplied by between two and nine myelinated nerve fibres which enter the base, side or top of the structure (Cauna, 1956). The myelin sheath is lost at the site of penetration or shortly after entering the corpuscle (Malinovsky, 1966). The nerve fibres divide extensively and course between the lamellar cells (Cauna, 1956). Immunohistochemical studies using antibodies against neurofilament protein and protein gene product 9.5, have revealed that the axons are arranged in loops which are orientated parallel to the epidermal surface (Dalsgaard *et al.*, 1984; Ramieri *et al.*, 1992; Vega *et al.*, 1996). As the axons course through the corpuscle they remain extracellular, but their membranes are in close contact with the membranes of the lamellar cells (Cauna and Ross, 1960). The axons have thin and thick segments along the length of their course. The thick regions contain accumulations of mitochondria and vesicles, whilst the thin segments contain a few neurofilaments (Castano *et al.*, 1995). The axons end in various specializations, the main ones being open networks, neurofibrillar end bulbs, terminal "boutons" and neurofibrillar varicosities (Cauna, 1956). Open networks of axons are formed by the extensive branching of afferent nerve fibres which then become entwined, thus forming a network of axons. Neurofibrillar end bulbs are swellings at the ends of axons

which have not undergone extensive branching. On the other hand, terminal "boutons" are swellings found on the ends of axons which have branched repeatedly. Neurofibrillar varicosities are terminal branches which have spindle-shaped swellings along their entire lengths. The axon terminals all contain dense collections of mitochondria, vesicles, glycogen granules, lipoidal material and neurofilaments (Halata and Munger, 1983). In many cases small axoplasmic processes containing fine filaments and clusters of vesicles arise from the axon terminals (Halata and Munger, 1983). In some parts of the corpuscle, cytoplasmic processes, from the lamellar cells, form concentric lamellae around the axon terminals (Cauna and Ross, 1960). Meissner's corpuscle is enclosed by a capsule composed of cells with elongated nuclei (Chouchkov, 1973). The cytoplasm of these cells contains well-developed rough endoplasmic reticulum cisternae, a prominent Golgi complex, free ribosomes, mitochondria and a few lysosomes which are located in the perinuclear region. Randomly orientated collagen fibres, embedded in an amorphous ground substance, occupy the spaces between the capsule and lamellar cells (Chouchkov, 1973).

In neonates, Meissner's corpuscles consist of coils of axons around a cluster of cells (Ridley, 1969). In rats the formation of Meissner's corpuscles occurs post-natally (Ide, 1977). In the early stages of development several axons are seen in contact with lamellar cells, similar in morphology to Schwann cells (Ide, 1977). The lamellar cells have numerous cytoplasmic processes which envelope the axons and attach the developing corpuscle to the basal lamina of the epidermis (Ide, 1977). Later the lamellar cells become horizontally stacked and the axons become flattened and arranged in loops (Ide, 1977).

COILED (KRAUSE) CORPUSCLES

Coiled corpuscles are similar to Meissner's corpuscles in structure and location. Coiled corpuscles supplied by between one and three nerve fibres

have been observed in the dermal papillae of pigs (Malinovsky *et al.*, 1982^b) and mice (Navarro *et al.*, 1995). The nerve fibres form intricate coils around lamellar cells. The nerve fibres in coiled corpuscles are orientated perpendicular to the epidermal surface, unlike in Meissner's corpuscles where the coils are orientated parallel to the epidermal surface (Halata and Munger, 1983). Coiled corpuscles contain nerve fibres which are round or oval in cross section, whilst those in Meissner's corpuscles have a flattened profile (Halata and Munger, 1983). The terminals of the coiled nerves are surrounded by several lamellae from the lamellar cells (Malinovsky *et al.*, 1982^b). The corpuscle is surrounded by a capsule which is continuous with the perineurium of the afferent nerve fibre (Malinovsky, 1986).

PACINIAN CORPUSCLES

Pacinian corpuscles are rapidly adapting mechanoreceptors stimulated by tissue deformation due to pressure. Pacinian corpuscles occur singly or in groups of up to five (Halata, 1977). The corpuscles are found closely associated with large blood vessels and arterio-venous anastomoses in the reticular layer of the dermis (Cauna and Mannan, 1958; Rettig and Halata, 1990; Kumamoto *et al.*, 1993). Pacinian corpuscles are also occasionally found within the perineurium of large nerves (Halata, 1977). The Pacinian corpuscle is composed of three regions: an inner core; an intermediate growth region and an outer bulb (Pease and Quilliam, 1957). The inner core is composed of bilaterally arranged lamellae which are formed by the extensive branching of cytoplasmic processes of cells located adjacent to the intermediate zone (Pease and Quilliam, 1957). Along the length of the corpuscle the two groups of lamellae are separated by longitudinal clefts (Pease and Quilliam, 1957). Using immunohistochemical techniques it has been shown that the lamellae forming

the inner core are immunoreactive for the glial specific protein S-100 which suggests that they are derived from Schwann cells (Iwanaga *et al.*, 1982; Hachisuka *et al.*, 1984, 1987; Vega *et al.*, 1996). In addition, a basal lamina envelopes the lamellar system of the inner core thus confirming their Schwann cell origin (Halata, 1977). The intermediate growth zone, located between the inner and outer zones, is composed of proliferating cells (Pease and Quilliam, 1957). The intermediate zone is only prominent in immature corpuscles. The outer bulb consists of concentrically arranged lamellae, formed by overlapping squamous cells (Shanthaveerappa and Bourne, 1963). The squamous cells are polyhedral in shape with marginally located nuclei and scant cytoplasm which contains a moderate amount of endoplasmic reticulum, in addition to a few mitochondria and Golgi cisternae (Cauna and Mannan, 1958). The squamous cells possess cytoplasmic processes which extend across the interlamellar spaces and contact cytoplasmic processes from neighbouring lamellae (Cauna and Mannan, 1958). Circularly arranged collagen fibres, blood vessels and round, "wandering" cells, with elongated nuclei and basophilic cytoplasm, are found in the interlamellar spaces of the outer bulb (Cauna and Mannan, 1958). Each Pacinian corpuscle is supplied by a single nerve fibre, the perineurium of which is continuous with the superficial lamellae of the outer bulb (Shanthaveerappa and Bourne, 1963). The nerve fibre loses its myelin sheath as it enters the corpuscle; soon after the Schwann cell sheath is also shed (Malinovsky, 1966). A space containing fibroblast-like cells separates the Schwann cell sheath from the inner core lamellae (Pease and Quilliam, 1957). Within the corpuscle, the axon is composed of pre-terminal, terminal and ultra-terminal regions (Breathnach, 1977). The pre-terminal region is the initial part of the axon, which is located outside the inner core, whilst the terminal and ultra-terminal parts are situated within the inner core. The elliptical-shaped terminal segment contains numerous peripherally located mitochondria, vesicles and

neurotubules, the centre of the axon being occupied by neurofilaments (Breathnach, 1977; Halata, 1977). The vesicles tend to be concentrated in the vicinity of the longitudinal clefts (Pease and Quilliam, 1957). Arising from the terminal segment are a few thin axoplasmic processes which extend into the longitudinal clefts on either side of the axon, where they end in terminal swellings (Cauna and Mannan, 1958; Zelena, 1978; Malinovsky *et al.*, 1982^a). The axoplasmic processes contain neurofilaments and clear vesicles which are situated directly beneath the axolemma (Malinovsky *et al.*, 1982^b). The greatest concentration of mitochondria in the terminal segment is situated below the axoplasmic processes, indicating that they are the region of the Pacinian corpuscle responsible for transduction (Bolanowski *et al.*, 1996). The axon ends in an expanded ultra-terminal segment which contains a large number of mitochondria and small vesicles (Pease and Quilliam, 1957). The ultra-terminal region has several axoplasmic processes which extend into the interlamellar spaces of the inner core (Halata, 1977; Bolanowski *et al.*, 1994).

Various forms of Pacinian corpuscles have been recognized in different species (Rettig and Halata, 1990). In some corpuscles the axon divides into two branches, each of which is enclosed in a separate inner core. As in more typical Pacinian corpuscles, the terminal and ultra-terminal segments contain numerous mitochondria and clear vesicles (Rettig and Halata, 1990).

Pacinian corpuscles are the first sensory receptors to develop in skin (Cauna and Mannan, 1959). The development of Pacinian corpuscles can be divided into three stages, primordial, avascular and vascular (Cauna and Mannan, 1959). In the primordial stage the corpuscle consists of a central branched axon surrounded by modified Schwann cells with short cytoplasmic processes which later form the inner core (Zelena, 1978). Pre-natally the lamellae of the inner core are loosely wrapped around the axon and are not bilaterally arranged (Zelena, 1978). In the early stages of development the cells

forming the inner core have lobulated nuclei and dense cytoplasm; the latter contains numerous free ribosomes, mitochondria and a moderate amount of rough endoplasmic reticulum (Zelena, 1978). During the avascular stage the axon loses its branches and dermal cells begin to form outer bulb lamellae. The vascular stage is characterized by blood vessels becoming trapped between the outer bulb lamellae formed by dermal cells. As the corpuscle matures the lamellae become thinner and more closely packed (Zelena, 1978). In the neonate, the corpuscle tends to be oval in shape with a thick, round distal end and a straight, tapered proximal end (Cauna and Mannan, 1958). The inner core adopts a hemiconcentric arrangement and the lamellae become tightly wrapped around the axon (Zelena, 1978). The cells of the inner core proliferate postnatally and are seen to contain several Golgi complexes adjacent to the nuclei (Zelena, 1978). With increasing age the corpuscle changes shape, becoming more irregular and tortuous (Cauna and Mannan, 1958).

BULBOUS (GOLGI-MAZZONI) CORPUSCLES

Simplified forms of Pacinian-like corpuscles are commonly seen in the papillary and reticular regions of the dermis. These corpuscles, known as bulbous or Golgi-Mazzoni corpuscles, usually occur in groups of up to nine (Cunningham and Fitzgerald, 1972; Malinovsky *et al.*, 1982^a; Rettig and Halata, 1990). In the goat, groups of bulbous corpuscles are found in the skin of the muzzle (Sar and Calhoun, 1966). Bulbous corpuscles have also been observed in the angle between sebaceous glands and hair follicles in sheep and the opossum (Lyne and Hollis, 1968; Halata, 1993). Similar corpuscles have also been reported in the horse (Talukdar *et al.*, 1970), cattle (Amakiri *et al.*, 1978), pig (Malinovsky *et al.*, 1982^b) and elephant (Rasmussen and Munger, 1996). The corpuscles consist of a branched, curved or twisted axon surrounded by

Schwann-like cells, one or more cytoplasmic lamellae and a perineural capsule (Fitzgerald, 1962; Cunningham and Fitzgerald, 1972; Malinovsky *et al.*, 1982^a). The myelin sheath is shed as the nerve fibre enters the corpuscle (Fitzgerald, 1962). Within the corpuscle the nerve fibre increases in diameter before ending in a straight or bent terminal which bears several axoplasmic processes (Cunningham and Fitzgerald, 1972). The interlamellar spaces contain collagen fibres embedded in an amorphous material (Rettig and Halata, 1990). Bulbous corpuscles begin development as enlarged sub-epidermal nerves surrounded by clusters of Schwann cells (Fitzgerald, 1962).

HAIR FOLLICLE INNERVATION

RUFFINI CORPUSCLE

The association between a Ruffini corpuscle and an hair follicle forms a structure known as a "pilo-Ruffini corpuscle" (Biemesderfer *et al.*, 1978). Ruffini corpuscles are slowly-adapting mechanoreceptors responding to tension in the connective tissue sheath surrounding the hair follicle (Halata, 1993). Ruffini corpuscles are located within the connective tissue sheath of hair follicles below the openings of sebaceous ducts (Biemesderfer *et al.*, 1978; Malinovsky, 1986). A layer of elastic fibres separates the Ruffini corpuscle from the epithelial cells forming the outer root sheath of the hair follicle (Halata, 1993). Ruffini corpuscles are innervated by large diameter ($5\mu\text{m}$) nerve fibres, from the superficial nerve plexus, which may course through the sebaceous gland before supplying the corpuscles (Stilwell, 1957; Bressler and Munger, 1983; Rettig and Halata, 1990). Within the connective tissue sheath the nerve fibres divide repeatedly before taking a circular course around the hair follicles (Munger and Halata, 1983; Halata, 1993). The axon terminals are wrapped in Schwann cell processes which in some cases form several concentric lamellae around each axon

(Malinovsky, 1986; Halata, 1993). In other areas Schwann cells only partially ensheath the axons, resulting in direct contact between the axons and surrounding collagen fibres (Byers, 1985). Each axon ends in a swelling containing numerous mitochondria, vesicles and neurofilaments (Schulze *et al.*, 1993a.). Schwann cell processes and septal cells divide the surrounding connective tissue into compartments (Bressler and Munger, 1983). Septal cells are elongated fibrocyte-like cells containing irregular-shaped nuclei and scant cytoplasm (Biemesderfer *et al.*, 1978; Bressler and Munger, 1983). The septal cell cytoplasm contains numerous pinocytotic vesicles.

PERIFOLLICULAR NERVE ENDINGS

Perifollicular nerve endings are thought to be rapidly-adapting mechanoreceptors which are stimulated by the movement of the hair shaft (Halata, 1993). Myelinated nerve fibres from the deep dermal nerve plexus lose their myelin sheaths as they approach the hair follicles (Halata, 1993). The nerves then divide into perifollicular nerve endings, which have a palisade arrangement around the hair follicles (Jenkinson and Blackburn, 1967; Bressler and Munger, 1983; Fundin *et al.*, 1995). The size and number of perifollicular terminals around each hair follicle is directly related to the size of the hair follicle (Rettig and Halata, 1990). The perifollicular terminals are inter-connected by short nerve fibres, suggesting that they function as a coordinated unit (Jenkinson and Blackburn, 1967). Each perifollicular nerve ending consists of a flattened axon sandwiched between two Schwann cell cytoplasmic processes (Munger and Halata, 1983; Halata, 1993). However, some perifollicular endings are enclosed by concentrically arranged Schwann cell lamellae (Halata, 1993). In typical perifollicular terminals, one edge of the flattened axon is embedded in the basal lamina of the hair follicle whilst the other edge is in contact with the

connective tissue sheath surrounding the hair follicle (Orfanos and Mahrle, 1973; Halata, 1993). The nerve endings contain numerous mitochondria and vesicles (Breathnach, 1977).

Perifollicular terminals possess axoplasmic processes which extend through the basal lamina of the hair follicle to contact the epithelial cells forming the outer layer of the hair follicle (Halata, 1993). The axoplasmic processes contain clear pinocytotic vesicles (Halata, 1993). No specializations of the axolemma or epithelial plasma membrane are seen at the points of contact between the axons and the outer root sheath epithelial cells (Breathnach, 1977). Loose connective tissue separates adjacent perifollicular endings (Rettig and Halata, 1990).

Early development of the perifollicular terminal is indicated by aggregations of Schwann cells around hair follicles below the level of the sebaceous gland ducts. In late gestation the perifollicular terminal consists of an irregular-shaped axon which is occasionally enveloped in Schwann cell cytoplasm (Bressler and Munger, 1983). At birth the perifollicular terminal has an adult-like morphology, with a flattened axon sandwiched between Schwann cells (Bressler and Munger, 1983).

FREE NERVE ENDINGS

It has been reported that a large proportion of free nerve endings are nociceptors. Other free nerve endings detect temperature changes, as well as touch and pressure (Guyton, 1991). Nerves ending blindly in the dermis have been identified with light and electron microscopes. In early light microscopy studies, the nerve endings were described as "free" because it was thought that they were not enveloped in Schwann cell sheaths (Breathnach, 1977). Electron microscopic studies have shown that these nerve endings are always completely or partially enveloped in a Schwann cell sheath or basal lamina.

(Breathnach, 1977). This has resulted in free nerve endings being redefined as nerve fibres unaccompanied by specialized structures, such as perineural cells. Within the papillary region, afferent nerve fibres lose their myelin sheaths and divide into several free nerve endings (Schulze *et al.*, 1993^a). Electron microscopic studies have shown that the point at which a free nerve ending branches from an afferent nerve fibre is marked by the presence of a terminal Schwann cell, which is characterized by the presence of abundant cytoplasm containing a well-developed Golgi complex, distended rough endoplasmic reticulum cisternae and some polyribosomes (Cauna, 1973; Cauna, 1980). Cytoplasmic processes from the terminal Schwann cell ensheathe the free nerve endings as they course towards the epidermis (Cauna, 1973). The afferent nerve fibre and free nerve endings are enclosed in a layer of collagen with fibres orientated in the same direction as the free nerve endings (Cauna, 1973). The free nerve endings are located in the sub-epidermal connective tissue or within the dermal papillae (Miller *et al.*, 1958; Jenkinson and Blackburn, 1967). On reaching the sub-epidermis the free nerve endings course through grooves on the under surface of the epidermis. The collagen sheath surrounding the free nerve endings is eventually lost, allowing direct contact between the basal lamina of the epidermis and the free nerve endings in areas where the Schwann cell sheath is incomplete (Cauna, 1973; Rettig and Halata, 1990). All the dermal papillae in the human hand contain free nerve endings (Miller *et al.*, 1958). Each dermal papilla contains as many as five free nerve endings formed by the branching of free nerve endings at the base of the papillae (Cauna, 1980). However, some free nerve endings course through the dermal papilla without branching (Cauna, 1980). Three structural forms of free nerve endings have been identified (Cauna, 1980). The first type are "open" free nerve endings which are incompletely enclosed by Schwann cell sheaths, resulting in wide areas being exposed to the basal lamina. The second type are "beaded" free

nerve endings which have spindle-shaped segments along their lengths, whilst the third type are "plain" free nerve endings which have tapered terminals undergoing little variation during their course. Ultrastructural studies have shown that the terminal regions of free nerve endings contain accumulations of mitochondria, clear vesicles, glycogen granules and neurofilaments (Halata and Munger, 1983; Schulze *et al.*, 1993a,b.).

MERKEL CELLS

The Merkel cell is thought to be a slowly-adapting mechanoreceptor which through its cytoplasmic processes and desmosomal connections with adjacent keratinocytes, detects deformities within the epidermis (Breathnach, 1980; Halata, 1993). In addition to being mechanoreceptors, Merkel cells are thought to have a multitude of additional functions. Merkel cell are thought to be neuroendocrine cells as they are immunoreactive for several neuroendocrine and neurochemical markers (Fantini and Johansson, 1995). The wide range of neuropeptides found in Merkel cells indicate that different subsets of Merkel cells exist (Moll *et al.*, 1995). In Merkel cells found in human skin various neurotransmitter markers, such as neurokinin A and somatostatin, have been identified.

Merkel cells may occur singly or in groups of more than two hundred cells (Rettig and Halata, 1990). Groups of Merkel cells are located in the stratum basale, at the base of epidermal ridges, and in the outer root sheath of hair follicles (Miller *et al.*, 1958; Halata and Munger, 1983; Halata, 1993; Fundin *et al.*, 1995). These groupings of Merkel cells correspond to highly sensitive areas of the skin (Tachibana, 1995). In histological sections the Merkel cell is identified as a large cell containing an irregular-shaped nucleus surrounded by vacuolated cytoplasm (Munger, 1965). Scanning electron microscopy studies

have shown that Merkel cells are ellipsoidal in shape with numerous cytoplasmic processes especially on the apical surface (Yamashita *et al.*, 1993). Ultrastructurally the Merkel cell is seen to contain a large lobulated nucleus, surrounded by cytoplasm which is less electron-dense than in adjacent keratinocytes (Lyne and Hollis, 1971; Halata and Munger, 1983). Rod-shaped inclusions are occasionally seen within the lobulated nucleus (Tachibana, 1995). The cytoplasm contains mitochondria, rough endoplasmic reticulum, free ribosomes, intermediate filaments and electron-dense granules. The intermediate filaments, which are perinuclear in location, occasionally form bundles (Tachibana, 1995). The electron-dense granules are thought to originate from the Golgi complex which is located on the opposite side of the cell (Munger, 1965; Lyne and Hollis, 1971; Breathnach, 1977; Tachibana, 1995). These granules, 80 to 120nm in diameter tend to be situated in the basal part of the cytoplasm and are thought to contain a neurotransmitter which is released when the cell is activated (Breathnach, 1980; Tachibana, 1995).

Merkel cells are separated from the basal lamina by cytoplasmic processes from adjacent keratinocytes (Chouchkov, 1974). Desmosomes connect the Merkel cells to adjacent keratinocytes (Lyne and Hollis, 1971; Schulze *et al.*, 1993^a). As the Merkel cells are attached to keratinocytes they move upwards as the outer layers of the epidermis are shed (Quilliam, 1978). The cytoplasmic processes have also been identified in ultrastructural studies and have been observed to extend between keratinocytes (Quilliam *et al.*, 1973; Schulze *et al.*, 1993^a; Tachibana, 1995).

Merkel cells are supplied by nerve fibres which lose their myelin sheaths in the sub-epidermal dermis, before entering the epidermis covered by a Schwann cell sheath (Rettig and Halata, 1990). The nerve then forms an expanded terminal which conforms to the shape of the Merkel cell (Munger,

1965; Ridley, 1969). A narrow gap separates the plasma membrane of the Merkel cell and the axolemma of the nerve terminal (Munger, 1965). Occasionally desmosome-like connections are seen between the plasma membrane of Merkel cells and the axolemma of nerve terminals (Chouchkov, 1974). The expanded nerve endings, which are termed "Merkel discs" by some authors, contain accumulations of mitochondria, lysosome-like electron-dense bodies, glycogen granules, vesicles and neurofilaments (Orfanos and Mahrle, 1973; Quilliam *et al.*, 1973; Chouchkov, 1974). Arising from the expanded terminal are thin axoplasmic processes which contain neurofilaments and vesicles (Chouchkov, 1974). Merkel cells are not always associated with nerve fibres (Tachibana, 1995; Hilliges *et al.*, 1996). It has recently been suggested that Merkel cells not associated with nerve fibres may have a paracrine function which leads to the stimulation of adjacent keratinocytes (Moll *et al.*, 1995).

Groups of expanded nerve endings terminating on Merkel cells are present at birth (Ridley, 1969). The origin and development of the Merkel cell is still unclear. Two hypotheses have been put forward to explain the origin of these cells. The first hypothesis suggests that Merkel cells differentiate from keratinocytes (Lyne and Hollis, 1971). This hypothesis is supported by the fact that Merkel cells resemble keratinocytes early in development and like keratinocytes, are linked to adjacent cells by desmosomes and are able to take up melanosomes (Lyne and Hollis, 1971; Tachibana, 1995). In addition, Merkel cells contain cytokeratin which indicates an epithelial origin (Moll *et al.*, 1986; Vielkind *et al.*, 1995). Recent research has shown that Merkel cells are derived from keratinocytes, but are unable to undergo cell division (Moll *et al.*, 1996). The second hypothesis suggests that Merkel cells have a neural crest origin. Hashimoto (1972) observed Merkel cells ensheathed by Schwann cells crossing the basal lamina of the epidermis from the dermis. As the Merkel cells entered the epidermis, the Schwann cell sheath was retracted to allow desmosomes to

form between the Merkel cells and adjacent keratinocytes. The Merkel cells then extended long processes into the dermis which were thought to draw nerve fibres into the epidermis (Moll *et al.*, 1995). This hypothesis of a neural crest origin is further supported by reports of migratory Merkel cells in the deep dermis (Moll *et al.*, 1995). In addition Merkel cells have been found to be immunoreactive for two neurospecific markers, protein gene product 9.5 and neurofilament protein, thus providing further evidence for a neural origin (Ramieri *et al.*, 1992; Fantini and Johansson, 1995). However, Merkel cells are not immunoreactive for S-100 protein which is a glial specific protein, indicating that these cells are unlikely to be derived from Schwann cells (Hachisuka *et al.*, 1984).

INTRAEPIDERMAL NERVES

In recent years the existence of intraepidermal nerves has been in doubt due to the unreliable light microscopic results obtained with silver impregnation and methylene blue staining techniques. In addition, the reports of intraepidermal nerves seen in ultrastructural studies have been disputed by Breathnach (1977) who reported that Merkel discs were the only nerves present in the epidermis. Recently, the use of immunohistochemistry in conjunction with electron microscopy, has conclusively demonstrated the existence of intraepidermal nerves throughout the epidermis (Hilliges *et al.*, 1995).

Intraepidermal fibres originate from small myelinated nerve fibres in the papillary region of the dermis (Wang *et al.*, 1990). The myelinated nerve fibres lose their myelin sheaths as they course towards the epidermis (Munger, 1965). Within the dermal papillae the nerve fibres form several branches which penetrate the basal lamina of the epidermis (Arthur and Shelley, 1959). In most cases the nerves enter the epidermis at the tip or sides of the dermal papillae (Ridley, 1969; Quilliam and Jayaraj, 1973; Hilliges *et al.*, 1996); however,

occasionally nerves enter the epidermis at the base of epidermal ridges (Munger, 1965). In some cases the nerve fibres supplying Meissner's corpuscles send branches into the epidermis (Novotny and Gommert-Novotny, 1988; Navarro *et al.*, 1995). In many cases intraepidermal nerves tend to enter the epidermis at a point near Merkel cells (Chouchkov, 1974). Kruger *et al.* (1981) have reported that the receptive area of intraepidermal nerves is located at the point where the nerve penetrates the basal cell layer. It is thought that the axons and Schwann cells penetrating the epidermis are mechanical nociceptors responding to intense, skin damaging mechanical stimulation.

After passing through the basal lamina the axons enter the epidermis by coursing through intercellular spaces between the keratinocytes, at which point they are ensheathed by interdigitating processes of both Schwann cells and keratinocytes (Kruger *et al.*, 1981). Once in the epidermis the axons may take a relatively straight course through the epidermis or may map irregular routes, sometimes forming loops around cells (Arthur and Shelley, 1959; Ramieri *et al.*, 1992). Most intraepidermal nerves are orientated perpendicular to the epithelial surface, but a few fibres may take a parallel route through the stratum spinosum or even form a wide loop and course back towards the basal lamina (Wang *et al.*, 1990; Kennedy and Wendelschafer-Crabb, 1993). The occurrence of branching within the epidermis is variable. Some workers (Ridley, 1969; Novotny and Gommert-Novotny, 1988) have reported rarely seeing nerves branching within the epidermis, whilst others, such as Jenkinson and Blackburn (1967), have reported the presence of extensive branching within the epidermis.

Most intraepidermal nerves end in the stratum granulosum, but a few thick fibres may extend as far as the stratum corneum (Ridley, 1969; Talukdar *et al.*, 1970). No anastomoses between nerve fibres have been observed within the epidermis (Miller *et al.*, 1958). Intraepidermal nerve fibres possess axoplasmic processes which contain neurofilaments, neurotubules and clear vesicles

(Chouchkov, 1974). In addition to axoplasmic processes, spindle-shaped swellings are often observed along the lengths of intraepidermal nerves (Quilliam and Jayaraj, 1973; Wang *et al.*, 1990; Hilliges *et al.*, 1996). These swellings contain masses of mitochondria, clear vesicles and lipoidal material (Munger, 1965). The intraepidermal nerves end in swollen terminals which are invaginated into the keratinocytes (Rettig and Halata, 1990). The swollen terminals contain numerous mitochondria and clusters of clear vesicles (Rettig and Halata, 1990).

Table 3.1..SENSORY RECEPTORS IN THE SKIN OF MAMMALIAN SPECIES.

<u>SPECIES</u>	<u>RECEPTOR</u>	<u>TECHNIQUE</u>	<u>REFERENCE</u>
Cattle:	Ruffini corpuscle;Merkel cell; Intraepidermal nerves;Pacinian corpuscle; Coiled corpuscle.	Silver impregnation.	Amakiri <i>et al.</i> ,1978
Goat:	Bulbous corpuscle. Perifollicular endings.	Silver impregnation. Methylene blue; Histochemistry.	Sar & Calhoun, 1966. Jenkinson & Blackburn, 1967.
Sheep:	Perifollicular endings. Merkel cell;Bulbous corpuscles.	Methylene blue Histochemistry Silver impregnation.	Jenkinson & Blackburn 1967. Lyne & Hollis,1968.
	Merkel cell; Intraepidermal nerves.	TEM.	Lyne & Hollis,1971.
	Merkel cell.	Silver impregnation.	Quilliam and Jayaraj, 1973.
Horse:	Free nerve endings; Intraepidermal nerves; Bulbous corpuscles.	Silver impregnation.	Talukdar <i>et al.</i> , 1970.
	Pacinian corpuscle.	Silver impregnation.	Bowker <i>et al.</i> ,1993.
Pig:	Bulbous corpuscles. Merkel cell; Intraepidermal nerves.	Methylene blue; Silver impregnation. TEM.	Fitzgerald, 1962. Quilliam <i>et al.</i> ,1973.
	Coiled corpuscle.	Silver impregnation TEM	Malinovsky <i>et al.</i> ,1982b..
	Merkel cell; Free nerve endings; Intraepidermal nerves; Ruffini corpuscle; Perifollicular endings.	Silver impregnation; TEM	Rettig & Halata, 1990.
Man:	Meissner's corpuscle.	Silver impregnation.	Cauna, 1956.
	Pacinian corpuscle; Ruffini corpuscle.	Methylene blue.	Stilwell, 1957.
	Pacinian corpuscle.	Silver impregnation.	Cauna & Mannan, 1958.
	Free nerve endings; Intraepidermal nerves;Merkel cell;Meissner's corpuscle.	Silver impregnation; Methylene blue.	Miller <i>et al.</i> , 1958.
	Intraepidermal nerves.	Methylene blue.	Arthur & Shelley,1959.
	Pacinian corpuscle.	Silver impregnation.	Cauna & Mannan,1959.
	Meissner's corpuscle.	TEM.	Cauna & Ross, 1960.
	Free nerve endings; Intraepidermal nerves.	Silver impregnation.	Ridley, 1969
	Merkel cell.	TEM.	Hashimoto, 1972.

Table 3.1..(continued)

<u>SPECIES</u>	<u>RECEPTOR</u>	<u>TECHNIQUE</u>	<u>REFERENCE</u>
Man:	Free nerve endings.	TEM.	Cauna, 1973.
	Meissner's corpuscle.	TEM.	Chouchkov, 1973.
	Merkel cell;Meissner's corpuscle;Perifollicular endings.	TEM; Histochemistry.	Orfanos & Mahrle, 1973.
	Meissner's corpuscle; Intraepidermal nerves; Merkel cell.	TEM.	Chouchkov, 1974.
	Merkel cell; Intraepidermal nerves; Meissner's corpuscle; Pacinian corpuscle; Perifollicular endings.	TEM.	Breathnach, 1977.
	Free nerve endings.	Silver impregnation; TEM.	Cauna, 1980.
	Meissner's corpuscle; Pacinian corpuscle.	Immunohistochemistry.	Iwanaga <i>et al.</i> , 1982.
	Free nerve endings; Intraepidermal nerves; Meissner's corpuscle.	Immunohistochemistry.	Dalsgaard <i>et al.</i> , 1984.
	Meissner's corpuscle; Pacinian corpuscle.	Immunohistochemistry.	Hachisuka <i>et al.</i> , 1984.
	Meissner's corpuscle;Merkel cell; Free nerve endings.	Immunohistochemistry.	Bjorklund <i>et al.</i> , 1986.
	Meissner's corpuscle.	Immunohistochemistry.	Hachisuka <i>et al.</i> , 1987.
	Intraepidermal nerves.	Silver impregnation.	Novotny & Gommert-Novotny, 1988.
	Intraepidermal nerves; Merkel cell; Meissner's corpuscle.	Immunohistochemistry.	Wang <i>et al.</i> , 1990.
	Intraepidermal nerves; Merkel cell; Meissner's corpuscle;Coiled corpuscle.	Immunohistochemistry.	Ramieri <i>et al.</i> , 1992.
	Intraepidermal nerves.	Immunohistochemistry.	Kennedy & Wendelschafer-Crabb, 1993.
Monkey:	Intraepidermal nerves.	Immunohistochemistry.	Castano <i>et al.</i> , 1995.
	Merkel cell.	Immunohistochemistry.	Fantini & Johansson, 1995.
	Merkel cell.	Immunohistochemistry.	Kim & Holbrook, 1995.
	Merkel cell	Immunohistochemistry.	Moll <i>et al.</i> , 1996.
	Ruffini corpuscle.	Silver impregnation; TEM.	Biemesderfer <i>et al.</i> , 1978.
	Meissner's corpuscle.	Silver impregnation.	Halata & Munger, 1983.

Table 3.1.. (continued)

<u>SPECIES</u>	<u>RECEPTOR</u>	<u>TECHNIQUE</u>	<u>REFERENCE</u>
Monkey:	Merkel cell; Free nerve endings; Ruffini corpuscle; Perifollicular endings.	Silver impregnation; TEM.	Bressler & Munger, 1983
	Ruffini corpuscle; Free nerve endings; Perifollicular endings; Merkel cell.	Silver impregnation; TEM.	Munger & Halata, 1983.
	Merkel cell.	Silver impregnation; TEM.	Dell & Munger, 1986.
	Intraepidermal nerves.	Immunohistochemistry.	Hilliges <i>et al.</i> , 1995.
Dog:	Merkel cell.	Immunohistochemistry; TEM.	Cheng-Chew & Leung, 1996
Cat:	Pacinian corpuscle;Bulbous corpuscle;Free nerve ending.	Methylene blue.	Malinovsky, 1966.
	Bulbous corpuscle.	Silver impregnation; Histochemistry.	Cunningham & Fitzgerald, 1972
	Intraepidermal nerves.	TEM	Kruger <i>et al.</i> , 1981.
	Pacinian corpuscle.	TEM	Malinovsky <i>et al.</i> , 1982.
	Pacinian corpuscle.	H&E.	Kumamoto <i>et al.</i> , 1993.
	Intraepidermal nerves; Merkel cell.	Immunohistochemistry.	Alvarez <i>et al.</i> , 1988.
	Merkel cell.	Immunohistochemistry; TEM.	Cheng-Chew & Leung,1996.
Rabbit:	Bulbous corpuscle.	Silver impregnation; Histochemistry.	Cunningham & Fitzgerald, 1972.
Rat:	Bulbous corpuscle.	Silver impregnation.	Cunningham & Fitzgerald, 1972.
	Pacinian corpuscle.	Histochemistry	Zelena, 1978.
	Meissner's corpuscle.	Immunohistochemistry.	Iwanaga <i>et al.</i> ,1982.
	Merkel cell.	SEM.	Yamashita <i>et al.</i> ,1993.
Mouse:	Pacinian corpuscle;Bulbous corpuscle;Perifollicular endings.	Immunohistochemistry.	Duc <i>et al.</i> , 1994.
	Bulbous corpuscle.	Silver impregnation; Histochemistry.	Cunningham & Fitzgerald, 1972.
	Meissner's corpuscle.	TEM.	Ide, 1977.
	Merkel cell.	Immunohistochemistry; TEM.	Pasche <i>et al.</i> , 1990.
Opossum:	Intraepidermal nerves.	Silver impregnation; TEM.	Munger, 1965.
	Merkel cell; Ruffini corpuscle; Perifollicular endings; Bulbous corpuscles.	Silver impregnation; TEM.	Halata, 1993.
Elephant	Pacinian corpuscles; free nerve endings.	Silver impregnation.	Rasmussen & Munger, 1996.

MATERIALS AND METHODS

ANIMALS

Seven bovine foetuses between 93 to 204 days of gestation, eight calves, ranging in age from 2 days to 4 weeks, and six kids, 10 hours to 4 weeks old, were used in the present study. Details of the foetuses, calves and kids are shown in Tables 3.2, 3.3 and 3.4.

Table 3.2. Details of bovine foetuses immunostained.

Estimated gestational age (days)	Crown-rump length (mm)	Sex
93	170	female
112	245	male
117	260	female
154	395	male
169	480	male
182	530	female
204	605	female

Table 3.3. Details of calves immunostained.

Age	Breed	Sex
2 days	Friesian	male
1 week	Friesian	male
2 weeks	Friesian	male
2 weeks	Friesian	male
3 weeks	Friesian	male
3 weeks	Friesian	male
4 weeks	Friesian	male
4 weeks	Friesian	male

Table 3.4. Details of kids immunostained.

Age	Breed	Sex
10 hours	Feral cross	male
2 weeks	Feral cross	male
3 weeks	Feral cross	male
3 weeks	Feral cross	male
4 weeks	Feral cross	male
4 weeks	Feral cross	male

INTRODUCTION TO NERVE STAINING

Early work on nerve demonstration used silver impregnation techniques and methylene blue staining to identify nerves and sensory nerve endings in skin (Samuel, 1953^a; 1953^b; Linder, 1978). These techniques were unreliable as they tended to stain connective tissue fibres in addition to the nerve fibres. Histochemical techniques, detecting acetylcholinesterase activity in sensory nerve endings, were later introduced to provide a more reliable procedure for demonstrating nerve fibres. These procedures, however did not stain smaller nerve fibres and tended to be strongly reactive for muscle fibres and blood vessels (Cunningham and Fitzgerald, 1972). Recently immunohistochemistry and electron microscopy have replaced histochemical procedures as the most reliable procedures for identifying nerves and sensory nerve endings (Shimizu *et al.*, 1996). The basis of immunohistochemistry is the ability of antibodies to bind to specific proteins, which are then made visible by the use of enzymes or fluorescent dyes (Monaghan and Moss, 1995). In the present study Bielschowsky's silver impregnation technique, the Avidin-Biotin-Peroxidase Complex immunohistochemical technique, and electron microscopy were used to identify nerve bundles and sensory nerve endings in the horn bud of calves and kids ranging in age from 10 days to 4 weeks.

LITERATURE REVIEW OF THE IMMUNOHISTOCHEMICAL TECHNIQUE

S-100 PROTEIN

Structure

Moore (1972) described S-100 as an acidic, low molecular weight (21,000- 24,000 daltons) protein. During the same year it was shown that S-100 was not a single protein, but was composed of three heterogeneous proteins which varied in amino acid composition and molecular weight (Stewart, 1972). Later, Isobe *et al.* (1977) confirmed that S-100 was formed by three separate proteins, but reported that two of the proteins were similar in amino acid composition and molecular weight; these proteins were termed phenylalanine-rich acidic protein 1a and phenylalanine-rich acidic protein 1b. The phenylalanine-rich acidic protein 1a and 1b components of S-100 were later renamed S-100 a and S-100 b (Baudier *et al.*, 1982). The third protein was identified as a calcium-binding protein (Baudier *et al.*, 1982; Vega *et al.*, 1996). The amino acid sequence of the proteins forming S-100 was further elaborated by Isobe and Okuyama (1978).

Tissue containing S-100

Moore (1972) noted that the brain contained 10 000 times more S-100 than any other organ. It was initially thought that S-100 was restricted to glial cells in the central nervous system (Perez *et al.*, 1970; Sviridov *et al.*, 1972; Matus and Mughal, 1975). This was the case until Haglid *et al.* (1974) demonstrated the presence of S-100 in the peripheral nervous system. Stefansson *et al.* (1982) later demonstrated the presence of S-100 in Schwann cells, and satellite cells of dorsal root and sympathetic ganglia. Kahn *et al.*

(1983) identified S-100 in a few non-neuronal cells, such as Langerhans cells and duct cells of the mammary gland.

Function and use of S-100

At the time when Moore (1972) described the characteristics of S-100, found in the brain, the role of S-100 was unknown, but it was thought that the protein was involved in the learning process. It was later shown that a proportion of S-100 was membrane-bound and able to bind to calcium (Rusca *et al.*, 1972; Hansson *et al.*, 1976). The ability of S-100 to bind calcium led to the suggestion that S-100 had a role in regulating the movement of calcium ions across plasma membranes (Isobe *et al.*, 1977). Hyden *et al.* (1980) observed that S-100 bound to calcium facilitated the movement of amino acids across plasma membranes. The role of S-100 was further expanded when Baudier *et al.* (1982) showed that S-100, bound to calcium, influenced the disassembly of microtubule proteins in the brain. Microtubules have been shown to play a role in the intracellular movement of organelles. Furthermore, Nika *et al.* (1982) have suggested that S-100 also has a role in calcium and potassium regulation.

Anti-serum to S-100 protein is used mainly in histopathology to demonstrate the presence of tumours, such as, meningiomas, Schwannomas (Winek *et al.*, 1989; D'Angelo *et al.*, 1991), and melanomas (Kindblom *et al.*, 1984; Carlson *et al.*, 1995). Recent reports have recognized S-100 anti-sera as a reliable marker for nerves and encapsulated nerve endings (Ramieri *et al.*, 1992; 1995).

NEUROFILAMENT PROTEIN

Neurofilaments are known as "intermediate filaments", because they have a diameter between that of small diameter actin filaments and large diameter

microtubules (Lazarides, 1980). Neurofilaments are composed of three polypeptides with molecular weights of 200 000, 150 000 and 70 000 daltons (Schlaepfer, 1977; Dahl, 1980).

Neurofilaments have been identified in neurons in both the peripheral and central nervous systems (Schlaepfer and Lynch, 1977). The neuronal cytoskeleton has been shown to consist of a lattice of neurofilaments, which are also thought to be involved in axonal transport (Hoffman and Lasek, 1975; Maeda *et al.*, 1987).

Anti-serum to the protein forming the neurofilaments was first produced by Dahl and Bignami (1977), using chick brain and human sciatic nerve as antigens. The anti-serum reacts specifically with all three neurofilament polypeptides (Dahl *et al.*, 1981). Naves *et al.* (1996) have shown that all sensory neurons are immunoreactive for all three neurofilament polypeptides. Neurofilament anti-serum has been used in neuron development studies (Raju and Dahl, 1982; Seiger *et al.*, 1984). Neurofilament anti-serum has also been used to study nerve degeneration and regeneration (Dahl and Bignami, 1978; Chi *et al.*, 1980). Due to its high specificity for neurons, neurofilament anti-serum has been extensively utilized to demonstrate fine nerve fibres in both the dermis and epidermis (Iwanaga *et al.*, 1982; Dalsgaard *et al.*, 1984; Bjorklund *et al.*, 1986; Yamamoto *et al.*, 1995).

AVIDIN-BIOTIN-PEROXIDASE COMPLEX

Principle of the technique

Hsu *et al.* (1981) introduced the Avidin-Biotin-Peroxidase Complex immunohistochemical procedure, which is a three-step, indirect technique used to demonstrate antigens in formalin-fixed, paraffin-embedded tissue (Figures 3.1-3.6). The procedure utilizes a primary antibody which binds to antigens in

either Schwann cells or axons (Figure 3.3). A biotinylated secondary antibody is then linked to the primary antibody (Figure 3.4), after which a pre-formed Avidin-Biotin-Peroxidase Complex is added (Figure 3.5). The peroxidase component of the Avidin-Biotin-Peroxidase Complex catalyzes a reaction between hydrogen peroxide and a chromogenic substrate, resulting in the visualization of the antigen's location (Figure 3.6). The high number of peroxidase molecules in the complex results in the Avidin-Biotin- Peroxidase Complex immunohistochemical technique being highly sensitive (Rose *et al.*, 1992).

Avidin

Avidin is a glycoprotein, with a molecular weight of approximately 68,000 daltons, which is found in egg-white (Newman and Hobot, 1993). Avidin has a very high affinity for biotin which was first revealed when it was discovered that rats fed egg-white developed symptoms of biotin (vitamin H or Coenzyme R-) deficiency (Griffiths, 1992). The binding between avidin and biotin occurs rapidly and has been shown to be virtually irreversible (Ormanns and Schaffer, 1985). The presence of four biotin binding sites on avidin enables it to act as a link between biotinylated immunoglobulins and enzymes (Griffiths, 1992). The form of avidin used in immunohistochemistry is streptavidin which is isolated from the fungus *Streptomyces avidinii* (Griffiths, 1992).

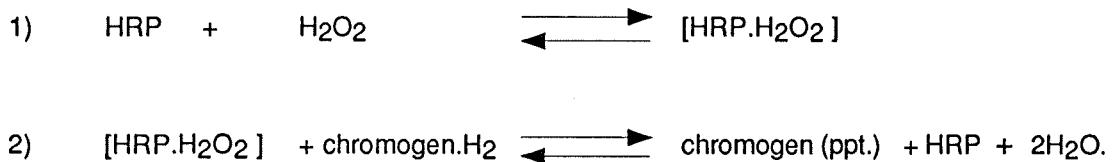
Biotin

Biotin is a low molecular weight vitamin which binds covalently to immunoglobulins and horseradish peroxidase (Hsu *et al.*, 1981). Biotin has been extracted from egg-yolk (Newman and Hobot, 1993). One of the early problems associated with the Avidin-Biotin-Peroxidase Complex technique was the binding of avidin to endogenous biotin which occurs in high concentrations

in liver, kidney, adipose tissue and mammary gland. Wood and Warnke (1981) solved this problem by devising a technique which detected and suppressed endogenous biotin thus increasing the sensitivity of the Avidin-Biotin-Peroxidase Complex technique.

Horseradish peroxidase

The enzyme horseradish peroxidase (HRP) is a hematin-containing glycoprotein which was originally isolated from the horseradish root (Mesulam, 1982). Horseradish peroxidase catalyses the reaction between hydrogen peroxide (H_2O_2) and chromogenic aromatic amines such as diaminobenzidine (Mesulam, 1982). The reaction is shown below:



AVIDIN-BIOTIN STAINING PROCEDURE

1. The developing horn bud and adjacent skin were cut into 1cm thick blocks which were then fixed in buffered neutral formalin for 5 days, after which they were processed and embedded in paraffin wax as described in the General Materials and Method chapter.
2. $5\mu m$ sections were cut, de-waxed in Histoclear and rehydrated through a series of alcohols to distilled water. The sections were then placed in Lugol's iodine for 5 minutes to remove traces of mercuric formol, before being placed in 2.5% sodium thiosulphate for a further 5 minutes. The

- sections were then rinsed in tap water.
3. The sections were placed in a 0.5% solution of hydrogen peroxide in methanol for 30 minutes to quench any endogenous peroxidase activity (Figure 3.1). The sections were then rinsed in water.
 4. The sections which were to be used to demonstrate neurofilament protein were placed in a Coplin jar containing 0.1M sodium citrate buffer pH 6 diluted 1:10 with distilled water. The sections were microwaved at 800 watts for 20 minutes to enhance neurofilament immunoreactivity. The sections were then rinsed in water.
 5. The slides were wiped to remove excess water, after which a ring was drawn around the outer margin of the sections with a PAP pen to help limit solution to the sections.
 6. Normal serum diluted with 10% Tris (hydromethyl) methylamine was poured onto the sections to block non-specific protein binding sites and thus reduce background staining (Figure 3.2). The normal sera used were obtained from the same species of animal as the secondary antibody. Normal goat serum was used on the sections to be stained for neurofilament protein, whilst normal horse serum was used on the S-100 sections. The serum was left on the sections for 30 minutes in a humidified chamber.
 7. The normal serum was tapped off and a primary antibody solution, diluted in 0.1% bovine serum albumin, itself diluted with 0.01% sodium azide in Tris (hydromethyl) methylamine, was poured on to the sections (Figure

- 3.3). The neurofilament sections were incubated with a 1:100 dilution of mouse anti-human neurofilament protein, whilst the S-100 sections were incubated at room temperature with a 1:1000 dilution of rabbit anti-bovine S-100, for 3 hours.
8. The primary antibody was then tapped off and the sections were washed for 3 minutes each in 3 changes of Tris(hydromethyl)methylamine-buffered saline.
9. The sections were then flooded with a biotinylated secondary antibody and incubated for 30 minutes in a humidified chamber (Figure 3.4). The neurofilament sections were incubated with goat anti-mouse serum, whilst the S-100 sections were incubated with horse anti-rabbit serum.
10. After 30 minutes the secondary antibody was tapped off and sections were washed for 3 minutes in 3 changes of Tris (hydromethyl) methylamine -buffered saline.
11. Avidin- Biotin-Peroxidase Complex reagent, from a "Vectastain Elite" kit, was poured on to the sections which were then incubated for 1 hour at room temperature (Figure 3.5).
12. The reagent was tapped off and the sections were washed in three changes of Tris(hydromethyl)methylamine-buffered saline, after which they were flooded with the chromogenic substrate, 3,3- diaminobenzidine tetrahydrochloride (Figure 3.6). The colour development was monitored under a light microscope and the substrate was washed off with water when the desired intensity of colour had been achieved.

13. The sections were then washed in water, counter-stained with haematoxylin, dehydrated and mounted in DFX. The slides were left on a hot tray overnight.

CONTROLS

Positive and negative controls were needed to test the specificity of the immunohistochemical procedure. The positive control was a section containing large nerve bundles. The negative control was a section containing nerve bundles which were not treated with the primary specific antibody.

PROBLEMS ENCOUNTERED

Formalin fixation of the tissue resulted in the masking of the neurofilament protein antigen resulting in a lack of nerve staining. The neurofilament antigen was retrieved by microwave heating the tissue sections in a sodium citrate buffer following the procedures of Shi *et al.* (1991; 1995). Although the microwave heating successfully retrieved the neurofilament antigen, the heating had a detrimental effect on some of the surrounding connective tissue.

MODIFIED BIELSCHOWSKY'S SILVER STAIN

1. 8 μ m paraffin sections were cut, dewaxed in Histoclear and then rehydrated through a series of graded alcohols to distilled water.
2. Sections were placed in 20% silver nitrate and kept in the dark at 37°C for 15 minutes. Sections were washed in three changes of distilled water for 2 minutes each change.
3. Concentrated ammonia solution was added to 50cm³ of silver nitrate until

the initial precipitation had disappeared. Sections were placed in this solution for 10 minutes at 37°C, after which they were washed in 0.1% ammonia.

4. 1cm³ of developer solution was added to the silver hydroxide solution.

The developer was made up as follows:

10% formalin	20cm ³
Distilled water	100cm ³
Concentrated nitric acid	1 drop
Citric acid	0.5g

Sections were left in the above solution for 5 minutes.

5. Sections were then washed in 0.1% ammonia followed by distilled water.
6. The sections were toned in 0.2% gold chloride for 10 seconds and then fixed in 5% sodium thiosulphate for 1 minute. Sections were then washed in tap water, dehydrated through graded alcohols, cleared in Histo-clear and mounted in DPX.

TRANSMISSION ELECTRON MICROSCOPY

Blocks of developing horn bud were obtained from bovine foetuses and calves. The tissues were processed as described in Chapter 1, General Materials and Methods.

LIGHT MICROSCOPY RESULTS

Neurofilament immunoreactivity was identified in nerve fibres in both the epidermis and dermis, as well as in Merkel cells in the epidermis. Intense S-100 immunoreactivity was demonstrated in Schwann cells and lamellar cells. Nerve bundles in the reticular dermis stained strongly with silver. However, few nerve fibres in the papillary dermis were silver stained.

CATTLE

DERMAL NERVES

At 93 days of gestation small nerves, often associated with blood vessels, were seen at the outer margins of the developing horn bud. A schematic representation of the different dermal layers is shown in Figure 3.7. These nerves were situated in the deep dermis directly above the cutaneous muscle layer. By 117 days of gestation the deep dermis was free of muscle, leaving only nerves in the deep dermal regions.

Post-natally nerve bundles, accompanied by thick walled blood vessels, were present in the lower region of the reticular dermis. In the 2-day-old calf each nerve bundle contained only a few fascicles. By 1-week of age the nerves had increased in size and contained as many as twenty fascicles. As the frontal process extended into the reticular dermis the nerves became marginalized to the periphery of the horn bud region. By 3 weeks of age no large nerves were found in the reticular dermis directly below the horn bud. However, small nerves composed of at least three fascicles were observed in the periosteum between the two bone masses forming the cornual process (see Chapter 5). The nerve bundles branched extensively within the periosteum and distributed nerves throughout the layer.

The internal structure of the nerve bundles located in the deep dermis consisted of Schwann cells and axons bound by perineurium. In the 93-day-old foetus most of the Schwann cells within the nerve bundles were negative for S-100. The nerve bundles were therefore identified by their location and by the presence of several axons enclosed in perineural connective tissue. At 117 days of gestation the Schwann cells contained eccentrically-located elongated nuclei which were negative for S-100. The nucleus was surrounded by intensely S-100-immunoreactive cytoplasm which contained several small, clear spaces which were presumed to indicate the location of axons. By 204 days of gestation the Schwann cell nucleus had become kidney-shaped and was located centrally in the cell. Abundant immunoreactive cytoplasm was located in the concave aspect of the nucleus, whilst only a thin rim of cytoplasm covered the convex side of the nucleus.

In calves less than 2-weeks old, each nerve fibre consisted of S-100-immunoreactive Schwann cell cytoplasm enclosing several, small round areas, which indicated the location of the axons. These round clear areas were more distinct than in the pre-natal nerve bundles. In the 4-week-old calves the S-100-immunoreactive cytoplasm formed a rim around a single, clear, irregular-shaped space in many of the nerve fibres, again indicating the location of an axon (Figure 3.8). In all the calves studied the axons were intensely neurofilament-immunoreactive and stained strongly with silver. The axons varied greatly in size and shape (Figure 3.9).

Bundles of axons and associated Schwann cells branched from the nerve bundles in the deep dermis and coursed obliquely through the reticular dermis accompanied by blood vessels. In the 93-day-old foetus these nerves were partially surrounded by a perineurium composed of cells with large, elongated nuclei. A continuous perineurium composed of several cell layers was seen at 117 days of gestation. Within the middle region of the reticular dermis, nerve

fibres branched from the oblique nerves and subsequently supplied structures in the papillary dermis. At 204 days of gestation a few arteriovenous anastomoses associated with small nerves were found between the hair follicles in the papillary dermis. The arterio-venous anastomoses were identified by the presence of thick-walled arterioles associated with thin-walled venules. A single layer of weakly S-100-immunoreactive nerve fibres encircled the arterio-venous anastomoses. In the 2-day-old calf, where the arteriovenous anastomoses were still relatively immature and few in number, one or two S-100-immunoreactive nerve fibres were seen surrounding the structures. These nerve fibres were not neurofilament-immunoreactive. The nerve fibres were enclosed in three to four layers of fibroblasts. By 1-week of age more arteriovenous anastomoses were observed in the middle reticular dermis than in the 2-day-old calf. The arterio-venous anastomoses were surrounded by intensely S-100 immunoreactive nerve fibres (Figure 3.10). In the 3 and 4-week-old calves the arterio-venous anastomoses had become more numerous and were distributed throughout the dermis. Numerous concentric nerve fibres enclosed each group of arterio-venous anastomoses. Next to each group of arterio-venous anastomoses were small nerve bundles consisting of fifteen to twenty nerve fibres. These small nerve bundles stained with silver and were S-100, as well as, neurofilament-immunoreactive.

CORPUSCULAR RECEPTORS

Coiled (Krause) corpuscles

Coiled corpuscles were rare, only being observed in one of the 2-week-old animals. The unencapsulated corpuscle consisted of a coiled nerve fibre associated with two lamellar cells which had large elongated nuclei. The coiled

nerve fibre was situated in the sub-epidermal dermis in contact with an epidermal ridge.

Bulbous (Golgi-Mazzoni) corpuscles

Two types of bulbous corpuscles were seen in the calves. It was unclear whether they were variants, or different stages of development of a single bulbous corpuscle. In all the calves studied small corpuscles were generally located in the papillary dermis, whilst larger corpuscles were found in the reticular dermis.

In the 2-day-old calf, the larger bulbous corpuscles were seen to consist of a thick central axon, a layer of presumptive lamellar cells and a capsule. The axon, which had thin and thick regions, ended in a brush-like terminal (Figure 3.11). By 1-week of age large bulbous corpuscles were seen to have developed S-100-immunoreactive cores which consisted of a mass of cytoplasmic processes. The immunoreactive core enveloped an elongated clear space which presumably indicated the location of the axon (Figure 3.12). A wide space separated the immunoreactive core from the capsule which was formed by cells with elongated nuclei. Also seen in the 1-week-old calf were smaller corpuscles in which the terminal axon was lined by lamellar cells with long cytoplasmic extensions. The ultra-terminal region of the axon was enclosed in a dense immunoreactive core formed by lamellar processes (Figure 3.13). The whole structure was enclosed in a capsule formed by cells with elongated nuclei. The cells forming the capsule were negative for S-100.

HAIR FOLLICLE INNERVATION

Ruffini corpuscle

At 93 days of gestation hair development was restricted to the centre of the horn bud where the epidermis was five to six cells thick. A few Schwann cells were seen in the vicinity of the developing hair germs, but none of them appeared to contact the hair germs. By 112 days of gestation nerve fibres were seen attaching to the neck regions of the developing hair follicles. Circularly arranged nerve fibres were first recognized at 182 days of gestation (Figure 3.14). These nerve fibres were the first indication of nerve terminal specialization and Ruffini corpuscle formation.

In the 2-day-old calf, hair follicles were seen throughout the horn bud region. Branches from the oblique nerves in the reticular dermis coursed parallel to the hair follicles. Side branches from these nerves formed circularly orientated profiles around the hair follicles, at the level of the sebaceous glands. The nerves ended in Ruffini corpuscles which were located between the sebaceous gland and hair follicle. The corpuscles consisted of groups of three to five Schwann cells and axons enclosed by connective tissue fibres and presumptive septal cells (Figure 3.15). The Schwann cells had strongly S-100 immunoreactive nuclei which were surrounded by a halo of clear cytoplasm which had an immunoreactive outer rim. Schwann cell nuclei were not visible in all sections; in some areas only immunoreactive cytoplasm containing small clear areas was seen (Figure 3.15). In sections stained for neurofilament protein the Ruffini corpuscles were seen to be formed by transversely sectioned axons which were located within the connective tissue sheath of the hair follicle (Figure 3.16). Two to three small axons were located in contact with presumptive Schwann cell nuclei. Groups of axons were also seen independent of the Schwann cell nuclei probably due to the nuclei missing the plane of section. The

Ruffini corpuscles were separated from the sebaceous gland by a layer of fibrocytes which marked the outer limit of the connective tissue sheath (Figure 3.16).

In the 1-week-old calf a few hair follicles were beginning to degenerate, but the Ruffini corpuscles were still intact. The degeneration in the 1-week-old calf was limited to pyknotic nuclei in a few of the hair follicles.

In the 3 and 4-week-old calves all the hair follicles were undergoing degeneration. The degenerating hair follicles were represented by disorganized masses of cells with vesicular nuclei. Circular nerve fibres could not be demonstrated with S-100 anti-sera. However, nerve fibres which were weakly neurofilament-immunoreactive were seen amongst some of the degenerating follicle cells (Figure 3.17). The typical Ruffini corpuscles formed by groups of Schwann cells, axons and septal cells were not visible.

Perifollicular nerve endings

Longitudinal nerves attaching to the epithelial cells of the hair follicle were seen at 204 days of gestation. The nerve fibres were seen in the upper part of the follicle below the level of the sebaceous ducts.

In the 2-day-old calf longitudinally orientated nerve fibres ended in perifollicular terminals which in transverse and oblique sections were seen to consist of rows of intensely immunoreactive Schwann cells with peripherally arranged nuclei (Figure 3.18). The Schwann cell cytoplasm formed broad processes which were in contact with the epithelial cells forming the outer root sheath of the hair follicle. Elongated clear areas, orientated perpendicular to the epithelial cells, were found between adjacent Schwann cells. The orientation and general location of the clear areas corresponded to the locations of axons stained for neurofilament protein (Figure 3.19).

In the 1-week-old calf hair follicles were beginning to show signs of degeneration. Schwann cell cytoplasmic profiles were weakly immunoreactive, and in transverse sections of the hair follicles the palisade arrangement of the perifollicular terminals was not seen in anti-S-100-stained sections. Instead a disarray of seemingly fragmented Schwann cell cytoplasmic processes was observed in the angle between the sebaceous gland and hair follicle. Some of the fragmented nerve fibres contacted the hair follicle.

By 2-weeks of age the hair follicles were at various stages of degeneration. Schwann cell profiles could not be identified in the region adjacent to the outer root sheath of the hair follicle. Instead the outer root sheath was enclosed in a weakly S-100-immunoreactive amorphous material. However, intact neurofilament-immunoreactive perifollicular axons were still present (Figure 3.20).

In the 2-day-old calf sweat glands were present at the base of the hair follicles. A few S-100-immunoreactive nerve fibres coursed through the connective tissue separating the glandular tubules, but no neurofilament-immunoreactive nerve fibres were seen. In addition, the sebaceous glands were innervated by nerve fibres which branched from the nerves running alongside the hair follicles. The nerve fibres coursed over the surface of the glands before continuing upwards to the uppermost region of the hair follicle. By 3 weeks of age the sebaceous glands had degenerated into disorganized masses of cells with pale nuclei. Isolated Schwann cell profiles, which were still intensely S-100-immunoreactive, were seen amongst these degenerating cells.

FREE NERVE ENDINGS

In the 112-day-old foetus branches from nerves which coursed obliquely through the dermis ended as free nerve endings in the sub-epidermal region (Figure 3.21). At this stage most of the free nerve endings terminated parallel to

the epidermal surface. At 154 days of gestation most of the free nerve endings were still orientated parallel to the epidermal surface, but a few nerves orientated perpendicular to the surface were also seen.

In all the calves studied, branches from the nerves which coursed between the hair follicles, extended towards the papillary dermis, where they divided into two or three pre-terminal nerves (Figure 3.22). These nerves were enclosed in cytoplasmic processes from Schwann cells with large, round, eccentrically placed nuclei which were intensely S-100-immunoreactive. Abundant cytoplasm enclosed the nucleus. The cytoplasm on one side of the nucleus was intensely S-100-immunoreactive, whilst the cytoplasm on the opposite side was weakly immunoreactive for S-100 (Figure 3.23). One or two of the nerve fibres extended close to the epidermis, where they divided into numerous free nerve endings which were unaccompanied by perineurial cells (Figures 3.22 & 3.23). These free nerve endings formed intensely neurofilament-immunoreactive, club-shaped terminals which contacted groups of Merkel cells located in the stratum basale (Figure 3.24). Although these free nerve endings were strongly neurofilament-immunoreactive, they were only weakly positive for S-100.

In the 2-day-old calf free nerve endings extended into the dermal papillae where they divided repeatedly to form a disorderly array of branches which had large swellings along their lengths. Some of these branches formed wide arcs parallel to the stratum basale cells at the apex of the dermal papillae. Branches from these nerves either ended in the dermal papillae, as tapered endings, or entered the epidermis. Terminal Schwann cells were regularly seen within the dermal papillae in the neonatal calf.

In the 4-week-old calves the number of free nerve endings within the dermal papillae was greatly reduced with only two to three unbranched nerve fibres seen in each dermal papillae (Figure 3.25). In all the calves studied it was

noted that there were fewer neurofilament-positive free nerve endings than S-100-immunoreactive endings within the dermal papillae.

MERKEL CELLS

In the 93-day-old foetus, Merkel cells with large, slightly indented nuclei and abundant clear cytoplasm were seen in the stratum basale in areas of hair germ initiation. No nerve fibres were seen attaching to these cells. By 112 days of gestation Merkel cells were seen within the outer root sheath of a few hair germs, as well as in the interfollicular epidermis. In some cases clusters of Merkel cells were found at the neck of the developing hair germs. Occasional nerve fibres were seen attaching to Merkel cells in the interfollicular epidermis. In the 182-day-old foetus several nerve fibres attached to Merkel cells which were located in the basal region of developing epidermal pegs (Figure 3.26).

In the 2-day-old calf the Merkel cells were characterized by the presence of large irregular-shaped nuclei which were paler than the nuclei of neighbouring keratinocytes (Figure 3.24). The Merkel cell nucleus was surrounded by faintly neurofilament-immunoreactive cytoplasm. Groups of four to five Merkel cells were observed in the stratum basale of epidermal pegs. Knob-like nerve terminals contacted the basal regions of these cells. Merkel cells were also seen in the stratum basale around the neck regions of hair follicles.

In the 1 and 2-week-old calves occasional Merkel cells were seen within the stratum spinosum over dermal papillae. In these calves several neurofilament-immunoreactive nerves were seen within the dermal papillae close to the Merkel cells, but no intraepidermal nerves were seen attaching to the cells. In the 3 and 4-week-old calves the Merkel cells had the same basic morphology as in the 2-day-old calf, except that melanin granules were present in the perinuclear regions of the cells.

INTRAEPIDERMAL NERVES

No intraepidermal nerves independent of Merkel cells were recognized pre-natally. In the 2-day-old calf, free nerve endings wrapped in Schwann cell cytoplasm were seen entering the epidermis at the tips of dermal papillae. In anti-S-100 stained sections some of the Schwann cell processes did not extend further than the stratum basale, whereas in others the Schwann cell processes extended as far as the middle layers of the stratum spinosum where they terminated in hook-like structures or simple tapered ends (Figure 3.27). Neurofilament-immunoreactive nerves were seen coursing as far as the stratum granulosum where they ended in plain or beaded terminals (Figure 3.28). Occasionally the neurofilament-immunoreactive swellings invaginated the stratum granulosum cells.

In the 2-week-old calves, free nerve endings entered the epidermis mainly through the sides of the dermal papillae or less commonly through the base of the epidermal pegs. These nerves were traced as far as the stratum spinosum where they terminated in plain endings. Occasionally the nerves extended close to the upper regions of the stratum granulosum where they ended in swellings.

By 4 weeks of age the majority of nerves entered the epidermis through the epidermal pegs. In these calves the Schwann cell sheath did not extend further than the stratum basale. Branching of the intraepidermal nerves was occasionally seen, especially within the stratum spinosum. Although most nerves took a relatively straight course through the epidermis, a few nerves formed arcs in the upper layers of the stratum spinosum.

GOATS

DERMAL NERVES

In the 10-hour-old kid, nerve fascicles accompanied by blood vessels were seen in the lower region of the reticular dermis. The internal structure of the nerve bundles was similar to that in calves, except for the presence of several small axons invaginated in each Schwann cell. In the 3-week-old kid the large dermal nerve bundles and blood vessels were located around the base of the cornual process.

In the 10-hour-old kid, arterio-venous anastomoses were seen in the papillary dermis. Three to four moderately S-100-immunoreactive nerve fibres encircled the arterio-venous anastomoses. By 2-weeks of age the number of nerve fibres encircling the arterio-venous anastomoses had increased and the nerve fibres were intensely S-100-immunoreactive. Arterio-venous anastomoses were seen in all layers of the dermis, with the larger, more complex vessels seen in deeper layers.

CORPUSCULAR RECEPTORS

No sensory corpuscles were identified in any of the kids studied.

HAIR FOLLICLE INNERVATION

Ruffini corpuscle

Nerve fibres arose from nerves accompanying arteriovenous anastomoses in the superficial regions of the reticular dermis. The nerve fibres gave off lateral branches which formed circular Ruffini corpuscles in the angles between sebaceous glands and hair follicles. The corpuscles consisted of groups of Schwann cells with centrally placed clear areas indicating the location

of the axons. Groups of fibroblast-like septal cells surrounded the individual nerve fibres. By 2-weeks of age most of the hair follicles in the horn bud region had degenerated, leaving a few cellular remnants. The remnants of the hair follicles were encircled by intensely S-100-immunoreactive nerve fibres (Figure 3.29). The Schwann cell nuclei as well as the cytoplasmic processes of these nerve fibres were still well defined. In later stages, fragmented moderately S-100-immunoreactive nerve fibres were seen associated with a few cells with pyknotic nuclei. The Schwann cell processes enclosed large, round vacuoles.

Perifollicular nerve endings

In the 10-hour-old kid, bundles of axons arose from nerves which coursed parallel to the hair follicles. The axons formed longitudinally orientated perifollicular endings which attached to the outer root sheath in the angle between the hair and sebaceous gland.

By 2-weeks of age perifollicular terminals in the horn bud centre had degenerated and could not be demonstrated immunohistochemically. However perifollicular terminals were still present around hair follicles at the margins of the horn bud. These terminals consisted of flattened axons enclosed on either side by cytoplasmic processes from the same Schwann cell (Figure 3.30).

FREE NERVE ENDINGS

In the 10-hour-old kid, nerve fibres from the nerve accompanying the arterio-venous anastomoses in the reticular dermis coursed into the dermal papillae. The nerves branched at the base of the dermal papillae into free nerve endings which extended into the papillae where they kept close contact with the stratum basale. A few of the free nerve endings continued into the epidermis through the apex and lateral walls of the dermal papillae (Figure 3.31).

Epidermal pegs, in the 10-hour-old kid, were innervated by sub-epidermal free nerve endings which coursed parallel to the base of the epidermal peg. The nerves either ended at the base of the epidermal peg or continued into the adjacent dermal papilla.

MERKEL CELLS

In the 10-hour-old kid, Merkel cells with nuclei surrounded by melanin granules and a halo of clear cytoplasm were seen in the epidermal pegs as well as in the stratum basale lining the dermal papillae (Figure 3.31). Nerve fibres attached to the basal regions of some of the Merkel cells in the epidermal pegs. In the 2 and 3-week-old kids the Merkel cells were devoid of melanin granules and had small dark nuclei. Most of the Merkel cells were associated with nerve fibres.

INTRAEPIDERMAL NERVES

Very few intraepidermal nerves were identified in kids. Occasional free nerve endings were seen entering the epidermis through the tips and sides of the dermal papillae. The Schwann cell cytoplasm enclosing the nerve fibres did not extend beyond the stratum basale. In the 10-hour-old kid intraepidermal nerves, still enclosed in Schwann cell cytoplasm, branched within the stratum basale (Figure 3.31).

ELECTRON MICROSCOPY RESULTS

DERMAL NERVES

Pre-natally, unmyelinated nerve bundles were seen in the horn bud dermis. The nerve fascicles consisted of groups of axons enclosed in Schwann cell cytoplasm. At 112 days of gestation the Schwann cells were only partially surrounded by basal lamina and were therefore identified as Schwann cells by their association with axons (Figure 3.32). The Schwann cell consisted of an elongated nucleus surrounded by cytoplasm, which contained rough endoplasmic reticulum, polyribosomes and a few vesicles. The invaginating axons were of different sizes and contained vesicles and filamentous material.

In calves aged between 2 days and 4 weeks, the nerve fascicles in the deep dermis of the horn bud consisted of both myelinated and unmyelinated nerve fibres (Figure 3.33). The myelin sheath and axons were of various shapes and sizes. Between the nerve fibres was endoneurium which consisted of collagen bundles and a few fibroblasts with elongated nuclei and electron-dense cytoplasm. Bundles of nerve fibres were enclosed in a perineurium consisting of at least three layers of cells with long lamellar processes. In the 4-week-old calves well-developed, unmyelinated nerves were seen surrounding the smooth muscle cells which formed the walls of the arterio-venous anastomoses (Figure 3.34). The unmyelinated nerves were composed of numerous axons which were filled with mitochondria, clear vesicles and a few electron-dense bodies.

A few sub-epidermal, nerves containing both myelinated and unmyelinated nerve fibres, were seen in the horn bud. The myelinated nerves consisted of axons packed with centrally-placed mitochondria and enclosed by a well defined myelin sheaths (Figure 3.35). The sub-epidermal nerves were

partially or fully enclosed in perineural sheaths. By 4-weeks of age, unmyelinated sub-epidermal nerves containing as many as twenty axons were seen.

CORPUSCULAR RECEPTORS

Bulbous (Golgi-Mazzoni) corpuscles

Bulbous corpuscles occurred singly in the papillary dermis of the 4-week-old calves. The myelinated pre-terminal nerve fibre entered the corpuscle through its lateral side. As it coursed through the corpuscle the nerve lost its myelin sheath. Several lamellar cells with irregular, elongated nuclei formed a few loose concentric lamellae around the terminal segment of the nerve fibre. A few axoplasmic processes from the terminal axon extended between the lamellae. The lamellar cells contained abundant cytoplasm which was filled with numerous mitochondria, polyribosomes and rough endoplasmic reticulum, all of which were diffusely distributed. The cytoplasmic processes which formed lamellae around the nerve fibre originated from different points of the lamellar cell. Numerous vesicles of various sizes and a few neurofilaments were seen in the lamellae.

The ultra-terminal segment was surrounded by up to ten closely aligned lamellae (Figures 3.36 & 3.37). The ultra-terminal part of the nerve fibre contained mitochondria which were distributed along the periphery of the axoplasm. Dense aggregations of neurofilaments were also present in the axoplasm. Mast-like cells with round, electron-dense bodies and long cytoplasmic processes enclosed the lamellae encircling the ultra-terminal region of the nerve fibre. The interlamellar spaces contained electron-dense amorphous material as well as collagen fibres. The whole structure was enveloped in a capsule which was made up of two layers of perineural cells with

elongated nuclei and long cytoplasmic processes.

A different form of bulbous corpuscle was identified in the horn bud reticular dermis of one of the 4-week-old calves. The single corpuscle was located close to a large nerve fascicle. The corpuscle consisted of two axonal profiles surrounded by collagen bundles. Two lamellae formed the inner core which was then partially surrounded by perineurial cells (Figure 3.38).

HAIR FOLLICLE INNERVATION

Ruffini corpuscle

In calves aged between 2 days and 2 weeks myelinated nerves divided repeatedly to form unmyelinated nerve fibres in the connective tissue surrounding hair follicles (Figure 3.39). The connective tissue sheath contained groups of four to ten axons enclosed in Schwann cell cytoplasm, as well as single axons covered by basal lamina. Some of the axons, which lacked a Schwann cell covering, contained numerous mitochondria; these axons were presumptive Ruffini terminals. Surrounding the groups of axon terminals were collagen bundles and presumptive septal cells which had elongated nuclei surrounded by cytoplasm which was drawn out into long processes (Figure 3.40). The cytoplasm contained distended cisternae. The septal cell cytoplasmic processes divided the surrounding collagen bundles.

Perifollicular nerve endings

Unmyelinated nerves were seen close to the hair follicles in calves aged between 2 days and 2 weeks. Within the connective tissue sheath surrounding the hair follicles were longitudinally-arranged axons, some of which were ensheathed in Schwann cell processes. In deeper sections, oblong and round

axons were observed close to the basal lamina surrounding the epithelial cells of the outer root sheath. The axons lacked cellular organelles, containing only a few fibrous clumps. Occasional Schwann cells, with large round nuclei and abundant cytoplasm filled with mitochondria, vesicles, rough endoplasmic reticulum and polyribosomes, were seen at the peripheral margin of the connective tissue sheath.

FREE NERVE ENDINGS

Free nerve endings were observed as early as 122 days of gestation in the horn bud dermis. At this stage of development, the free nerve endings were seen coursing parallel to the epidermal surface in the papillary dermis directly below Merkel cells. In bovine foetuses, aged between 122 and 206 days, the free nerve endings contained elongated mitochondria, regularly-arranged dense aggregations of neurofilaments, electron-dense granules and peripherally-located vesicles. The free nerve endings were partially enveloped in Schwann cell cytoplasm which contained a large amount of rough endoplasmic reticulum and numerous electron-dense bodies of various sizes.

Post-natally free nerve endings were identified in all the calves studied. At 2-days of age, branched free nerve endings were seen in the sub-epidermal region (Figure 3.41). The nerves varied greatly in diameter. The thinner free nerve endings contained few organelles, occasional vesicles and a scattering of neurofilaments. The thicker free nerve endings contained large centrally located vesicles, surrounded by a few neurofilaments. The thicker nerves tended to be only partially enveloped by Schwann cell sheaths. The areas of the free nerve ending not covered by Schwann cell processes were covered only by basal lamina. The Schwann cell processes contained several small pinocytotic vesicles, in addition to a few electron-dense bodies. Basal laminae covered the Schwann cell processes. By 2-weeks of age, several extensively branched free

nerve endings were seen close to the epidermal ridges. Two or three large pre-terminal nerves, partially enclosed by perineurium, branched from afferent nerves in the papillary dermis. The point of origin of the pre-terminal nerves was marked by the presence of a terminal Schwann cell with a large, round nucleus surrounded by electron-dense cytoplasm. At 4 weeks of age the terminal Schwann cell had a large nucleus surrounded by abundant cytoplasm which contained numerous mitochondria, rough endoplasmic reticulum and polyribosomes at one end of the cell (Figure 3.42). The concentration of organelles on one side of the cell resulted in a region of greater electron density. The other end of the cell contained a few rough endoplasmic cisternae and polyribosomes, resulting in a relatively electron-lucent cytoplasm. In all animals the Schwann cell cytoplasm was drawn out into processes which partially enclosed the pre-terminal nerves. The pre-terminal nerves contained a few neurofilaments and occasional vesicles. As the nerves coursed towards the epidermis, they branched extensively to form numerous free nerve endings close to the epidermis. Unlike the pre-terminal nerves, the free nerve endings contained several mitochondria and vesicles. The free nerve endings were wrapped in a Schwann cell sheath which originated from the terminal Schwann cell. The Schwann cell sheath was filled with bundles of neurofilaments orientated parallel to the epidermis. A few mitochondria and electron-dense bodies were also present. Close to the epidermis the free nerve endings were only partially covered by a Schwann cell sheath. Many of the axons were only enveloped in a basal lamina.

Dermal papillae in the horn bud were innervated by myelinated nerve fibres which lost their myelin sheaths at the base of the papillae. Once the myelin sheath had been shed, the axon increased greatly in size to form a dilated ending which was filled with numerous mitochondria as well as parallel arrays of neurofilaments (Figure 3.43). A terminal Schwann cell formed concentric

lamellae around the dilated axon. The terminal Schwann cell cytoplasm contained rough endoplasmic reticulum, lipoidal material, mitochondria and polyribosomes. Surrounding the dilated axon were a few unmyelinated nerves and free nerve endings which continued into the dermal papilla. The dilated axon, Schwann cell and surrounding nerve branches were surrounded by a discontinuous perineurium through which the free nerve endings entered the dermal papilla.

MERKEL CELLS

In the 198-day-old foetus, Merkel-like cells containing highly indented nuclei surrounded by electron lucent cytoplasm were seen in the stratum basale. The cytoplasm in the perinuclear area contained polyribosomes, rough endoplasmic reticulum and a few clear vesicles. Basally the cytoplasm extended into a long process which protruded through the basal lamina to contact a Schwann cell in the dermis (Figure 3.44 A & B). The cytoplasmic process terminated in an expanded end which contained numerous ribosomes. A narrow gap separated the presumptive Merkel cell from the Schwann cell which was partially enclosed by basal lamina. The Schwann cell was associated with an axon which was located close to the stratum basale.

Elsewhere in the horn bud of the 198-day-old foetus, Merkel cells were identified by the presence of indented nuclei, basally-located electron-dense bodies and several cytoplasmic processes (Figure 3.45). The electron-dense bodies were membrane-bound and were scattered amongst several mitochondria in the basal region of the cell, as well as in some cytoplasmic processes. Occasional intraepidermal nerves were seen making contact with the Merkel cell cytoplasmic processes which contained large electron-dense bodies. However, no membrane specializations were seen at the points of contact. In foetuses aged between 203 and 206 days, Merkel cells were seen above the

first layer of stratum basale cells, as well as in contact with cells forming the stratum intermedium. The Merkel cells in the supra-basal position were associated with intraepidermal nerves which contained clumps of glycogen, a few neurofilaments and large vesicles. Several small desmosomes were seen linking the Merkel cells to adjacent cells.

INTRAEPIDERMAL NERVES

In the horn bud intraepidermal nerves were not recognized pre-natally. However, post-natally in one of the 2-week-old calves, a few intraepidermal nerves were observed. Most of the intraepidermal nerve profiles were found in intercellular spaces, although occasional intraepidermal nerves appeared to invaginate the keratinocytes (Figure 3.46). The morphology of the nerves varied. In some cases axons with a thick axolemma enclosing a disorganized mass of filamentous material were seen. In other cases axons containing mitochondria, vesicles and electron-dense bodies were observed.

DISCUSSION

One of the aims of this study was to provide an immunohistochemical and ultrastructural description of the sensory innervation of the horn bud area in both calves and kids. The results of the present study indicate that the early post-natal innervation of the horn bud is similar to that of hairy skin. Hairy skin typically has numerous free nerve endings and few corpuscles. The present study has established that post-natally the horn bud is supplied by a dense, well-developed nerve network resulting in it being a very sensitive structure. This is supported by observations that calves disbudded without anaesthesia exhibit signs of pain, such as struggling and vocalization (Hemsworth *et al.*, 1995;

Bengtsson *et al.*, 1996). In addition, it has been shown that dehorning is associated with stress, which is most likely caused in part by the pain inflicted on the animal (Boandl *et al.*, 1989; Wohlt *et al.*, 1994; Morisse *et al.*, 1995; Petrie *et al.*, 1996).

DERMAL NERVES

In the present study it was observed that in bovine foetuses less than 204 days old the Schwann cell cytoplasm was immunoreactive, whilst the nucleus remained negative. The Schwann cell cytoplasm was seen to enclose several small spaces which indicated the locations of the invaginating axons. This suggests that pre-natally nerve bundles are predominantly composed of unmyelinated nerves consisting of several axons invaginated into a single Schwann cell. This was supported by electron microscopy which showed groups of unmyelinated axons invaginating Schwann cells. There do not appear to be any reports in the literature regarding the immunoreactivity of developing nerves. However, there are reports on the ultrastructure of developing nerves in human foetuses (Gamble and Breathnach, 1965; Breathnach, 1977). The electron microscopic appearance of pre-natal nerves in the current study was similar to observations of developing nerves in human foetuses (Gamble and Breathnach, 1965). As in the study by Gamble and Breathnach (1965), the Schwann cells seen in the dermis underlying the bovine horn bud had elongated nuclei surrounded by cytoplasm which contained prominent rough endoplasmic reticulum and vesicles. Gamble and Breathnach (1965) reported the occasional presence of groups of axons enclosed by cytoplasmic processes from two Schwann cells; such complexes were not seen in any of the bovine foetuses studied.

During the development of nerves Schwann cells divide and progressively separate the axons into smaller bundles until a one to one ratio between Schwann cell and axon is achieved (Breathnach, 1977). In the post-natal calves less than 2-weeks old the S-100 positive Schwann cells still enclosed several axons. By 3 weeks of age a one-to-one relationship between Schwann cells and axons had been achieved in most of the nerve bundles. Immunohistochemical studies of this 3-week age group revealed that the Schwann cell cytoplasm formed a rim around a single, irregular-shaped area. The morphology of these Schwann cells resembled those found in human skin (Hachisuka *et al.*, 1984). Hachisuka *et al.* (1984) proposed that the large clear area enclosed by Schwann cell cytoplasm contained an axon and myelin sheath, both of which were negative for S-100. In the present study the ultrastructural appearance of the nerve fibres corresponded with the irregular profiles demonstrated with immunohistochemistry. In addition, the clear areas revealed with anti-S-100 sera were seen to correspond with irregular-shaped axons demonstrated with anti-neurofilament sera.

In all the animals studied bundles of axons branched from the deep dermal nerve bundles and coursed obliquely through the reticular dermis to form a superficial nerve plexus in the papillary dermis. This branching pattern is similar to that observed in human (Wang *et al.*, 1990) and cattle skin (Amakiri *et al.*, 1978). Branches from the superficial nerve network supplied arterio-venous anastomoses. As early as 204 days of gestation the arterio-venous anastomoses were innervated by S-100 positive nerve fibres. The S-100 protein is located in Schwann cell cytoplasm. As the arterio-venous anastomoses and nerves matured the intensity of S-100 immunoreactivity increased. In all cases the nerves surrounding the arterio-venous anastomoses were negative for neurofilament protein which is found in axons. These results suggest that S-100 immunoreactivity is found in both sensory and autonomic nerve fibres, whilst

neurofilament protein is found only in sensory nerves. These findings are similar to immunohistochemical observations of sensory and autonomic nerves in human skin (Bjorklund *et al.*, 1986). Bjorklund *et al.* (1986) were able to show that S-100 occurred in nerve fibres which were also positive for known autonomic neurotransmitters. Although it is not possible to positively classify nerves as being autonomic on the basis of their ultrastructure, the presence of numerous small vesicles in unmyelinated nerve fibres strongly suggests that they are autonomic (Orfanos and Mahrle, 1973). In the current study numerous vesicles were seen within the axons of unmyelinated nerves encircling arterio-venous anastomoses. These unmyelinated nerves corresponded with S-100 positive nerves seen with the light microscope. Associated with the arterio-venous anastomoses were nerve bundles which were immunoreactive for both S-100 and neurofilament. These nerves are probably a mixture of sensory and autonomic nerve fibres, the sensory fibres innervating the hair follicles, whilst the autonomic nerve fibres innervate the arterio-venous anastomoses and sweat glands.

CORPUSCULAR RECEPTORS

Coiled (Krause) corpuscles

Coiled corpuscles were rarely encountered in the horn bud region of the calves studied and were not seen in these regions in any of the kids. In the calves the corpuscle consisted of a loose coil of nerve fibres around presumptive lamellar cells. Unlike the coiled corpuscles observed in the pig's snout (Malinovsky *et al.*, 1982^b), the corpuscles in the present study were situated beneath epidermal pegs rather than within dermal papillae. Malinovsky *et al.* (1982^b) recognized two forms of coiled corpuscle which varied in the number of nerve fibres present, as well as in the extent of coiling. The corpuscles identified

in the current study correspond to the simpler form of corpuscle recognized in the pig, in which a single loosely coiled axon was seen (Malinovsky *et al.*, 1982^b). In the present study the coiled corpuscles lacked capsules, unlike the corpuscles observed in the pig (Malinovsky *et al.*, 1982^b). Coiled corpuscles are thought to be functionally similar to Meissner's corpuscles, which are known to be rapidly adapting mechanoreceptors (Malinovsky, 1986).

Bulbous (Golgi-Mazzoni) corpuscles

Several forms of bulbous corpuscles were identified in the current study. In the 2-day-old calf bulbous corpuscles consisted of a central straight axon surrounded by lamellar cells. Similar corpuscles have been observed in the skin of the mouse, rat, rabbit, cat (Cunningham and Fitzgerald, 1972) and horse (Talukdar *et al.*, 1970). Cunningham and Fitzgerald (1972) described the bulbous corpuscles as consisting of a central axon surrounded by cells which were reactive for acetylcholinesterase. Meanwhile, the corpuscles in the pig's snout (Quilliam *et al.*, 1973) resembled the small bulbous corpuscles seen in the 1-week-old calf in the present study, which consisted of an axonal terminal region lined by lamellar cells, whilst the ultra-terminal segment was surrounded by a dense core of lamellae. Electron microscopic examination of these corpuscles showed them to consist of a thick central axon surrounded by concentric lamellae. The lamellae were formed by lamellar cells which lined the terminal and ultra-terminal segments of the axon. The occurrence of similar lamellated corpuscles has been noted in the opossum (Halata, 1993). Halata (1993) reported the presence of axoplasmic processes from the ultra-terminal part of the axon extending between the lamellae. In the present study axoplasmic processes were not observed in the ultra-terminal segment although a few were seen branching from the terminal region of the axon. The presence of

numerous vesicles of various sizes was noted in the lamellae of corpuscles in the present study. This is in agreement with observations of lamellae in the opossum, where the vesicles have been described as being pinocytotic (Halata, 1993).

It has been reported in the pig and the marsupial, *Monodelphis domestica*, that the inner core of bulbous corpuscles is formed by hemiconcentric lamellae separated by two longitudinal clefts (Rettig and Halata, 1990; Schulze *et al.*, 1993^b). However, in the present study a distinct hemiconcentric lamellar arrangement was not seen. The interlamellar spaces contained an electron-dense amorphous material as well as collagen fibres. This is in agreement with reports in the pig (Rettig and Halata, 1990). In the calves studied, mast-like cells were seen in contact with the lamellae surrounding the ultra-terminal axon. The mast-like cells contained round electron-dense bodies, but lacked the numerous villous processes associated with mast cells. Heine and Forster (1975) have observed mast cells in close contact with unmyelinated pre-terminal nerves in the dog. They proposed that the mast cells participated in a neuro-hormonal feedback mechanism involving the nervous and blood systems.

In the 4-week-old calves studied, the lamellae were enclosed in a capsule formed by flattened perineurial cells with long cytoplasmic processes. This correlates well with reports of bulbous corpuscles in the marsupial, *Monodelphis domestica* (Schulze *et al.*, 1993^b).

Apart from typical lamellated bulbous corpuscles, simplified corpuscles were recognized in the reticular dermis. The structure of these corpuscles resembled that of simple lamellated corpuscles observed in the anal canal of the pig (Rettig and Halata, 1990).

HAIR FOLLICLE INNERVATION

Ruffini corpuscles

As reported by Bressler and Munger (1983) the first stage of Ruffini corpuscle formation is the aggregation of Schwann cells close to the hair germs. This is in agreement with observations in the present study in which groups of Schwann cells were seen in the vicinity of developing hair germs at 93 days of gestation. Pre-natally Ruffini corpuscles could not be recognized, although circularly-arranged nerve fibres were seen in the early part of the third trimester. It has been proposed that nerves are involved in hair germ initiation and development (Bressler and Munger, 1983). However the exact mechanism is still unclear.

Distinct Ruffini terminals were seen within the connective tissue sheath surrounding the hair follicles in calves aged between 2 days and 1 week. The terminals were formed by the extensive branching of myelinated nerves. The branching pattern of nerves in the present study is similar to the pattern observed in the monkey (Biemesderfer *et al.*, 1978). However, in contrast to finding by Biemesderfer *et al.* (1978), the Ruffini corpuscles in the present study were not surrounded by perineurial cells.

Neurofilament studies showed the presence of two or three small axons associated with each Schwann cell. Sections stained for S-100 revealed immunoreactive Schwann cell cytoplasm with clear areas, presumably containing axons. Similarly, electron microscopy showed the presence of at least four axons per Schwann cell. The structure and position of Ruffini terminals in the present study was similar to Ruffini terminals seen around primate facial hairs (Bressler and Munger, 1983). As in the present study, the Ruffini corpuscles in the primate consisted of small axons surrounded by Schwann cell cytoplasm. Groups of these unmyelinated nerves were embedded in connective

tissue which was then separated into compartments by presumptive septal cells. The morphology of septal cells in the present study resembles that of septal cells found around primate facial hairs (Biemesderfer *et al.*, 1978). As in the primate, the septal cells in the present study had long cytoplasmic processes.

As the hair follicles degenerated the characteristic Ruffini corpuscle structure was lost. In the 3 and 4-week-old calves S-100 immunoreactivity was lost before neurofilament immunoreactivity. Unlike in calves, intact S-100 immunoreactive circular nerves were still present in advanced stages of hair follicle degeneration in kids. In later stages the Schwann cell enclosed large clear areas which were assumed to be degenerating swollen axons.

Perifollicular terminals

Although longitudinal nerves were seen in the bovine foetuses, no typical perifollicular terminals were identified. In contrast, Bressler and Munger (1983) identified perifollicular terminals attaching to the outer root sheath of hair follicles in pre-natal primates.

In the present study, perifollicular terminals were recognized post-natally in both calves and kids. In calves the axons were sandwiched between the cytoplasmic processes of adjacent Schwann cells, whereas in kids axons were placed between two cytoplasmic processes from the same Schwann cell. The perifollicular terminals in kids resembled those described in primate facial hairs (Bressler and Munger, 1983), and in the snout of the opossum (Halata, 1993).

FREE NERVE ENDINGS

The current study has demonstrated both neurofilament and S-100 immunoreactivity in free nerve endings in the dermis below the developing horn bud in calves and kids. The presence of neurofilament immunoreactivity in free

nerve endings is in line with findings in the human where neurofilament protein occurs in sensory nerves, but not in autonomic nerves (Dalsgaard *et al.*, 1984; Bjorklund *et al.*, 1986). The presence of neurofilament protein only in sensory nerves may be the reason why fewer neurofilament reactive than S-100 reactive free nerve endings were observed in the present study. However the presence of S-100 immunoreactivity in free nerve endings is contrary to reports by Bjorklund *et al.* (1986). The presence of neurofilament-immunoreactive fibres in human skin led Dalsgaard *et al.*, (1984) to suggest that these nerves are responsible for the detection of several sensations such as touch, heat and pain. If this is the case the horn bud regions of both calves and kids are highly sensitive regions early in life.

The present study has established that free nerve endings develop early in the bovine foetus resulting in a well-developed morphology at birth. Ultrastructurally definitive free nerve endings were first seen at 122 days of gestation. The nerves contained elongated mitochondria, regular arrays of neurofilaments, electron-dense granules and vesicles. This description is in general agreement with that given by Bressler and Munger (1983) on free nerve endings supplying primate facial hairs. However, in primates the neurofilaments lacked an organized arrangement. The free nerve endings in the present study were partially enclosed in a Schwann cell sheath which was not present in the free nerve endings seen by Bressler and Munger (1983).

Post-natally tufts of immunoreactive free nerve endings innervated the epidermal ridges and dermal papillae. The structure of the terminal tree of free nerve endings, close to the epidermal ridges, is in agreement with observations of free nerve endings in human skin (Cauna, 1973). Cauna (1973) investigated the branching pattern of free nerve endings by making reconstructions of serial sections viewed with the electron microscope. The immunohistochemical procedures used in the present have made it possible to

observe the branching pattern of free nerve endings without the use of serial sections and graphic reconstructions. The position at which the nerve fibres branch from afferent nerves is marked by the presence of a terminal Schwann cell which has a morphology different to that of typical Schwann cells. The ultrastructure of the terminal Schwann cells in the present study differs from that described in human skin (Cauna, 1973). The terminal Schwann cells observed by Cauna (1973) lacked the eccentrically-placed accumulation of mitochondria observed in the present study. The accumulation of mitochondria resulted in one side of the cell being more electron-dense than the other side. The intense immunoreactivity seen on one side of the terminal Schwann cells is most likely due to this accumulation of mitochondria.

The terminals of the free nerve endings close to the epidermal ridges were weakly S-100 positive, implying a very thin investment of Schwann cell cytoplasm around each axon. Electron microscopy confirmed that the nerve endings close to the epidermis were only partially covered by a thin Schwann cell sheath, thus leaving wide areas of axon in direct contact with the surrounding connective tissue. The presence of wide areas of axon devoid of Schwann cell envelope is one of the characteristic features of free nerve endings (Halata and Munger, 1983; Rettig and Halata, 1990). As the terminal regions of the free nerve endings were not clearly defined with anti-S-100 sera, anti-neurofilament sera was used to demonstrate the termination of free nerve endings on Merkel cells in the stratum basale. The nerve terminals formed irregular knob-like structures which contacted the basal regions of the Merkel cells. The presence of expanded nerve terminals below Merkel cells has been observed in the opossum (Halata, 1993), and pig (Rettig and Halata, 1990).

Free nerve endings forming terminal trees were not seen in the kids studied; instead single nerve fibres were seen coursing parallel to the epidermal pegs in close contact with the stratum basale. Similar nerves have been

described in human skin (Wang *et al.*, 1990). Unlike in the calves, terminal Schwann cells were not identified in the kids. In calves terminal Schwann cells were almost exclusively associated with extensively branched nerve fibres which were not seen in kids. Free nerve endings in some of the calves originated from dilated axons situated at the basal ends of the dermal papillae. The mode of branching and the presence of terminal Schwann cells at the point of branching are in line with observations made by Cauna (1980) on interpapillary nerves in human digital skin. However, in the present study the terminal Schwann cells formed concentric lamellae around the dilated axons. In this respect the axons resembled the ultra-terminal regions in bulbous corpuscles. However, unlike the bulbous corpuscles, unmyelinated nerves, formed by several axons invaginating Schwann cell cytoplasm, were situated within the bounds of the perineurium. In addition, the morphology of the terminal Schwann cells differed from that of the lamellar cells found in bulbous corpuscles. Terminal Schwann cells had an eccentric concentration of organelles, whilst in lamellar cells the organelles were diffusely distributed in the cytoplasm.

Regardless of the location or species, free nerve endings are characterized by the presence of numerous mitochondria, vesicles and neurofilaments (Halata and Munger, 1983; Munger and Halata, 1983; Rettig and Halata, 1990; Schulze *et al.*, 1993^a). In the 2-day-old calf, S-100 positive free nerve endings composed of spindle-shaped segments were seen. The presence of thin and thick segments along the course of free nerve endings was confirmed with the electron microscope. The thick segments were seen to contain an accumulation of mitochondria, whilst the thin regions contained few organelles. The occurrence of enlargements along the free nerve endings has been reported in the cat (Heppelmann *et al.*, 1990). According to Heppelmann *et al.* (1990), the spindle-shaped enlargements represent receptive sites for painful

stimuli.

MERKEL CELLS

The observations in the present study indicate that the appearance of Merkel cells coincides with hair germ initiation. The association of Merkel cells with developing hair germs has been noted in fetal sheep epidermis (Lyne and Hollis, 1971).

In both light and electron microscopic studies the characteristic morphology of the Merkel cell, with indented nuclei and clear cytoplasm, was apparent. With the electron microscope, the Merkel cell cytoplasm was less electron-dense than in neighbouring keratinocytes. This is a characteristic feature of Merkel cells in most species, although in the early part of gestation the Merkel cell cytoplasm is similar in density to that of adjacent keratinocytes (Lyne and Hollis, 1971). Lyne and Hollis (1971) noted electron-dense bodies in Merkel cells in sheep foetuses ranging in age from 57 to 144 days of gestation. This is contrary to findings in the current study in which presumptive Merkel cells, with indented nuclei and electron-lucent cytoplasm appeared lacking in electron-dense bodies. These cells were presumed to be Merkel cells because they made contact with Schwann cells situated in the dermis. The basal lamina was seen to be discontinuous on either side of the presumptive Merkel cell. This observation supports the hypothesis proposed by Hashimoto (1972) that Merkel cells from the dermis are able to break the basal lamina and migrate into the epidermis where they form desmosomal contacts with adjacent keratinocytes.

In many Merkel cells identified in the current study, contacts with axons were seen. This corresponds with findings by Pasche *et al.* (1990) who estimated that between 50 and 95% of Merkel cells in developing mouse epidermis were associated with nerve endings. Pasche *et al.* (1990) further

suggested that the Merkel cells served as targets for intraepidermal nerve development during embryogenesis.

In the present study Merkel cells were neurofilament-immunoreactive, but negative for S-100. The presence of neurofilament protein in Merkel cells has previously been noted in the human (Fantini and Johansson, 1995). In the present study neurofilament immunoreactivity was noted throughout the Merkel cell cytoplasm. This is contrary to findings by Fantini and Johansson (1995) in which neurofilament immunoreactivity was concentrated in the basal regions of the Merkel cells.

INTRAEPIDERMAL NERVES

In the present study, intraepidermal nerves ensheathed by Schwann cell processes were revealed with anti-S-100 sera. This is contrary to observations in the human, where no Schwann cell cytoplasmic processes were seen in the epidermis (Ramieri *et al.*, 1992). In both calves and kids, S-100 positive Schwann cell processes were seen extending beyond the basal lamina into the stratum basale. In calves the Schwann cell processes extended as far as the middle layer of the stratum spinosum, whereas in kids the cytoplasmic processes did not extend beyond the stratum basale. The presence of axons ensheathed in Schwann cell processes within the stratum spinosum has been described as a rare occurrence (Breathnach, 1977). It is generally accepted in the literature that the Schwann cell process is shed as the nerve crosses the basal lamina (Chouchkov, 1974). Chouchkov (1974) has reported that as the nerves enter the epidermis they become ensheathed by keratinocytes in the same manner that they are wrapped in Schwann cell processes in the dermis.

In calves less than 2-weeks old, as well as in all the kids, free nerve endings entered the epidermis mainly through the sides and apical regions of the dermal papillae. Later, as terminal sprays of free nerve endings developed,

most nerves entered the epidermis through the epidermal pegs. The latter situation correlates well with observations of nerves entering the epidermis in the cat (Alvarez *et al.*, 1988). Likewise, in the human, free nerve endings enter the epidermis mainly through the epidermal pegs (Hilliges *et al.*, 1995).

In the present study intraepidermal nerves took a relatively straight course through the epidermis, although occasional tortuous arrangements were observed. This is in agreement with observations in human skin in which the course of the nerves was variable (Wang *et al.*, 1990; Kennedy and Wendelschafer-Crabb, 1993). The intraepidermal nerves in the horn bud extended into the stratum spinosum or granulosum where they ended in tapered or swollen terminals. The occurrence of swollen terminals has been well documented (Alvarez *et al.*, 1988; Hilliges *et al.*, 1995). The swollen terminals have been shown to correspond to areas of nerve filled with mitochondria and vesicles (Rettig and Halata, 1990). In the current study few mitochondria or vesicles were seen in the intraepidermal nerves.

SUMMARY

INNERVATION OF HORN BUD

1. A few bulbous corpuscles were recognized in the reticular dermis of the calves studied. However, no corpuscular receptors were observed in any of the kids studied.

2. The earliest free nerve ending was observed in a 112-day-old bovine foetus. Initially the free nerve endings terminated parallel to the epidermal surface, whereas later they were orientated perpendicular to the surface.

3. In the post-natal calves studied free nerve endings divided extensively to form sprays of small branches which terminated on epidermal cells. Such terminal sprays were not observed in any of the kids studied.
4. Merkel cells, characterized by clear or neurofilament-immunoreactive cytoplasm, were identified in both calves and kids. In calves the Merkel cells were concentrated in the basal regions of epidermal ridges, whilst in kids the Merkel cells were found in both the epidermal ridges and dermal papillae walls.
5. In both calves and kids free nerve endings ensheathed in Schwann cell cytoplasm entered the epidermis. In most cases the Schwann cell sheath was lost within the stratum basale.

INNERVATION OF HAIR FOLLICLES

1. The first sign of hair follicle innervation was seen at 93 days of gestation in the bovine. At this stage Schwann cells were seen close to developing hair follicles, but none of the nerves appeared to make contact with the hair follicles.
2. By 112 days of gestation several nerves terminated on the hair follicles.
3. Nerves arranged circularly around hair follicles were first observed at 182 days of gestation, whilst longitudinally orientated nerves were seen at 204 days of gestation.
4. Well-developed perifollicular and Ruffini terminals were seen soon after

birth in both calves and kids.

5. The structure of perifollicular and Ruffini terminals was lost as the hair follicles in the horn region degenerated. In the 3 and 4-week-old calves studied perifollicular and Ruffini terminals were absent.

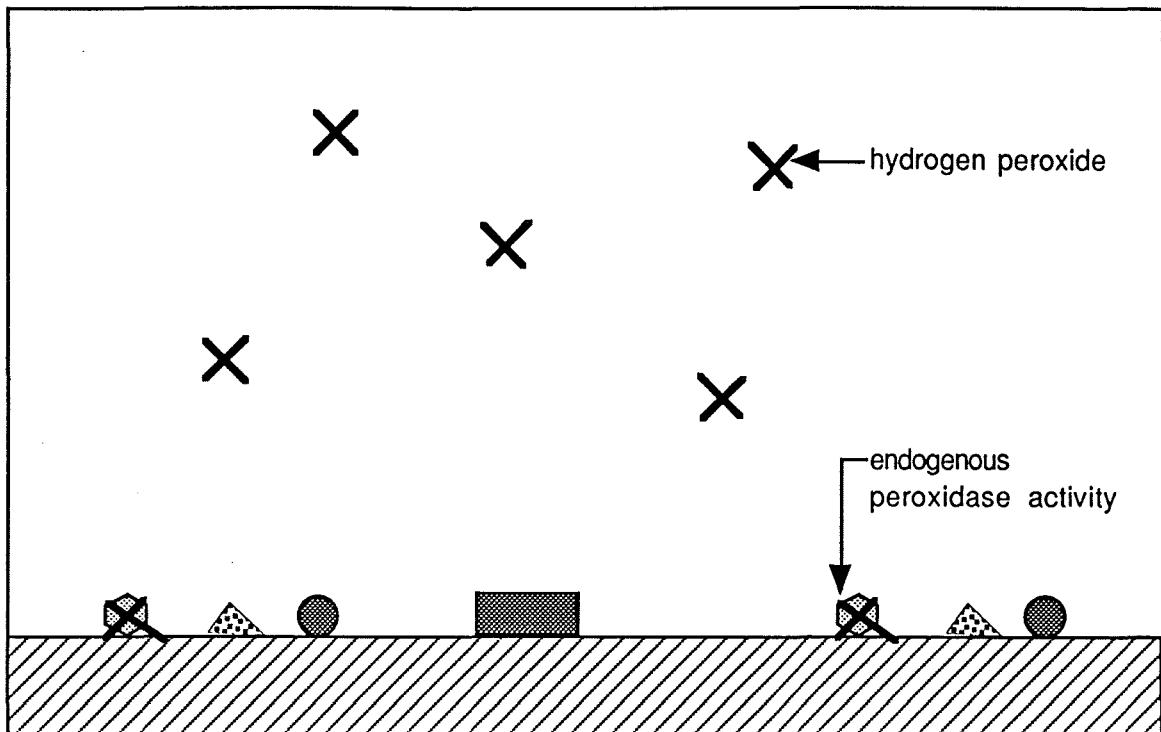


Figure 3.1. Endogenous peroxidase activity is blocked by adding hydrogen peroxide.

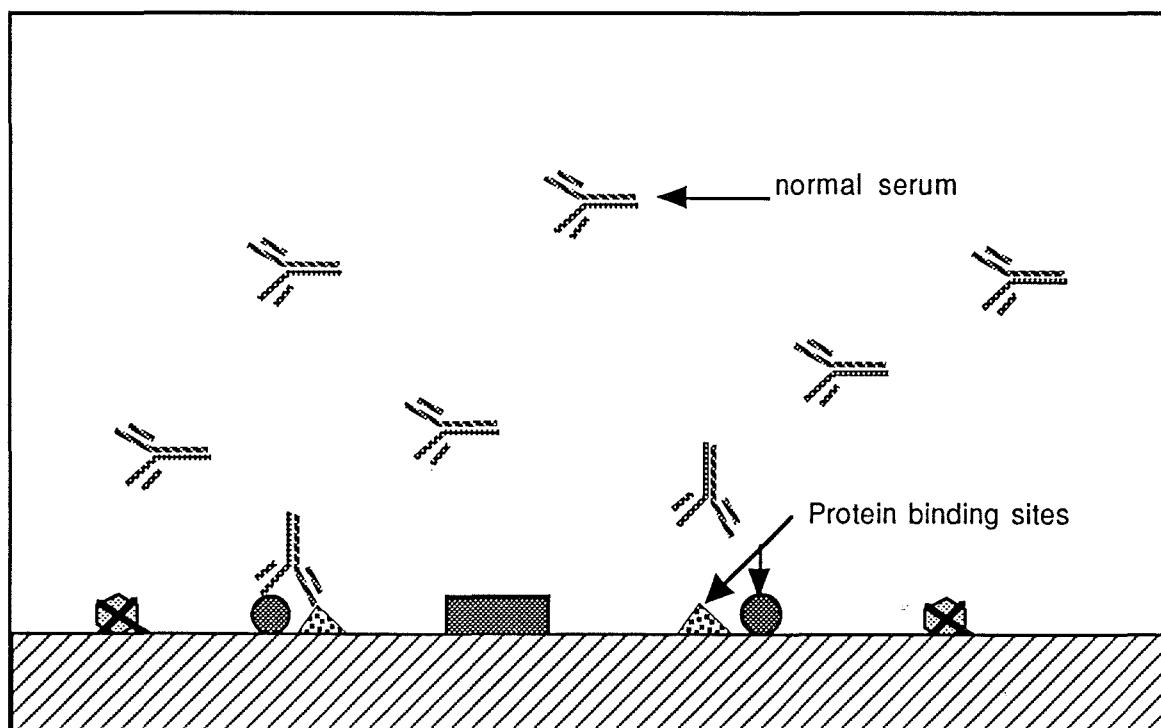


Figure 3.2. Add normal serum to block non-specific protein binding sites.

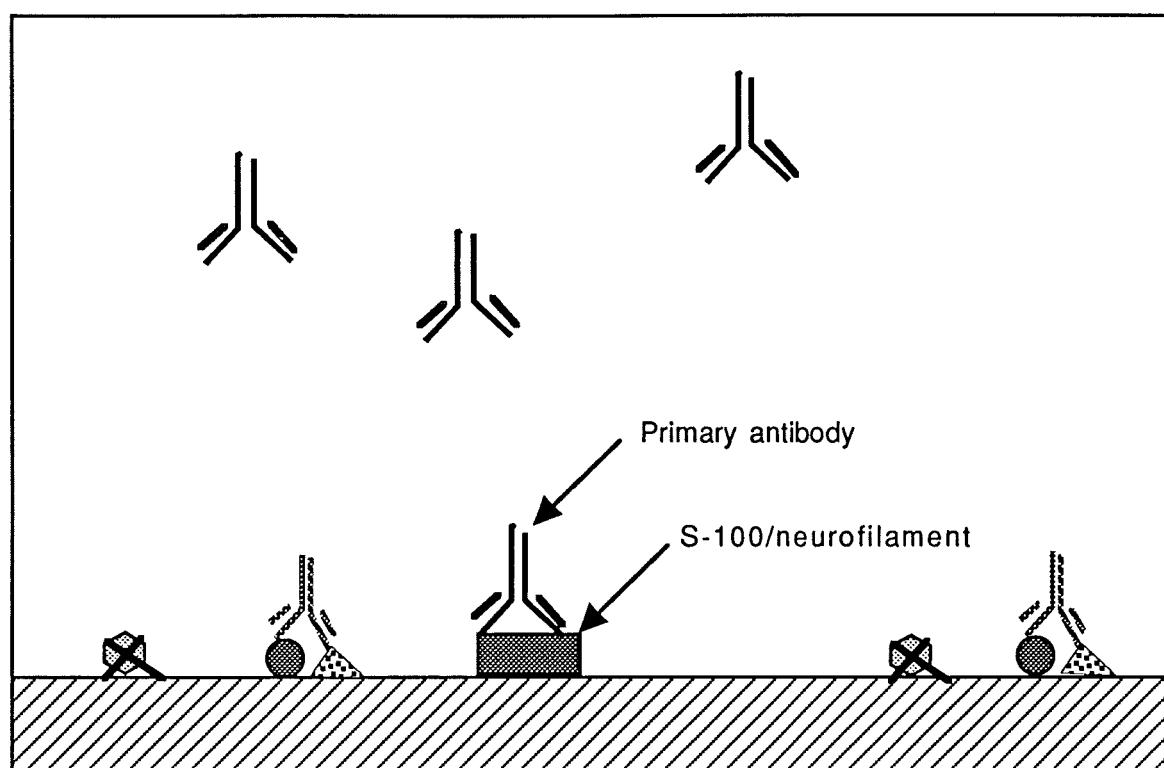


Figure 3.3. The primary antibody (rabbit anti-bovine S-100/ mouse anti-human neurofilament) attaches to S-100 or neurofilament protein in Schwann cells and neurons respectively.

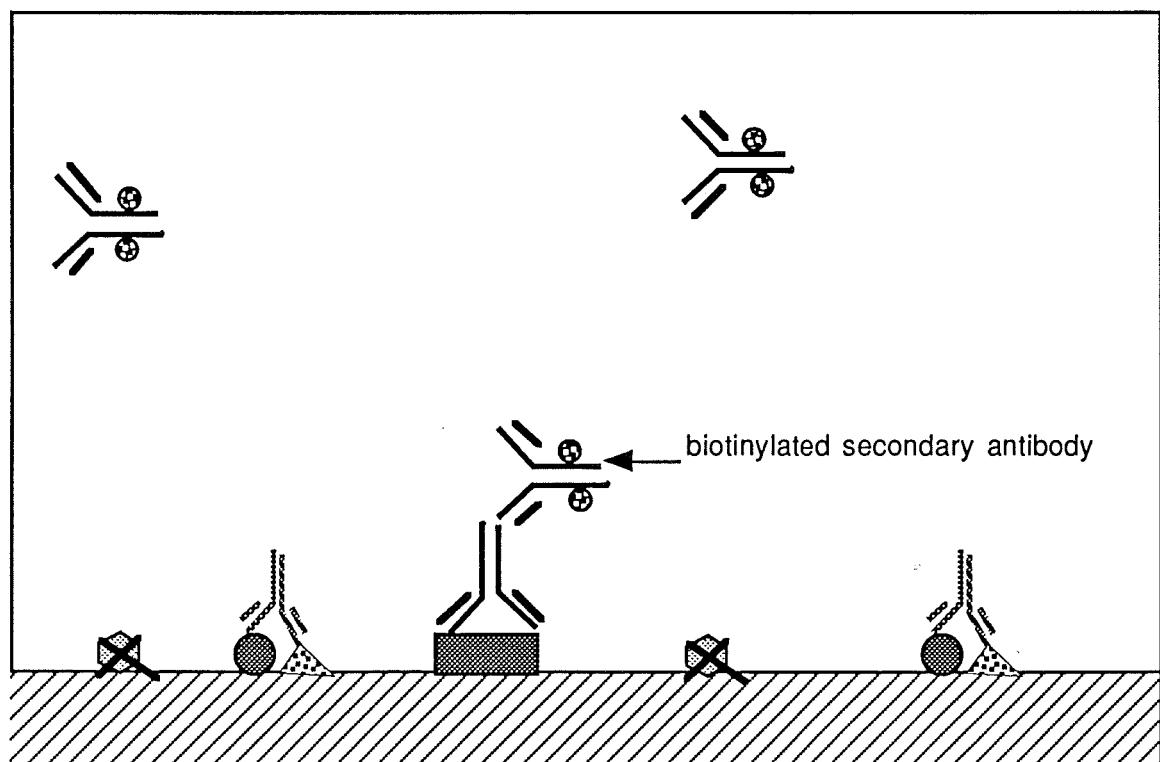


Figure 3.4. Biotinylated secondary antibody attaches to the primary antibody.

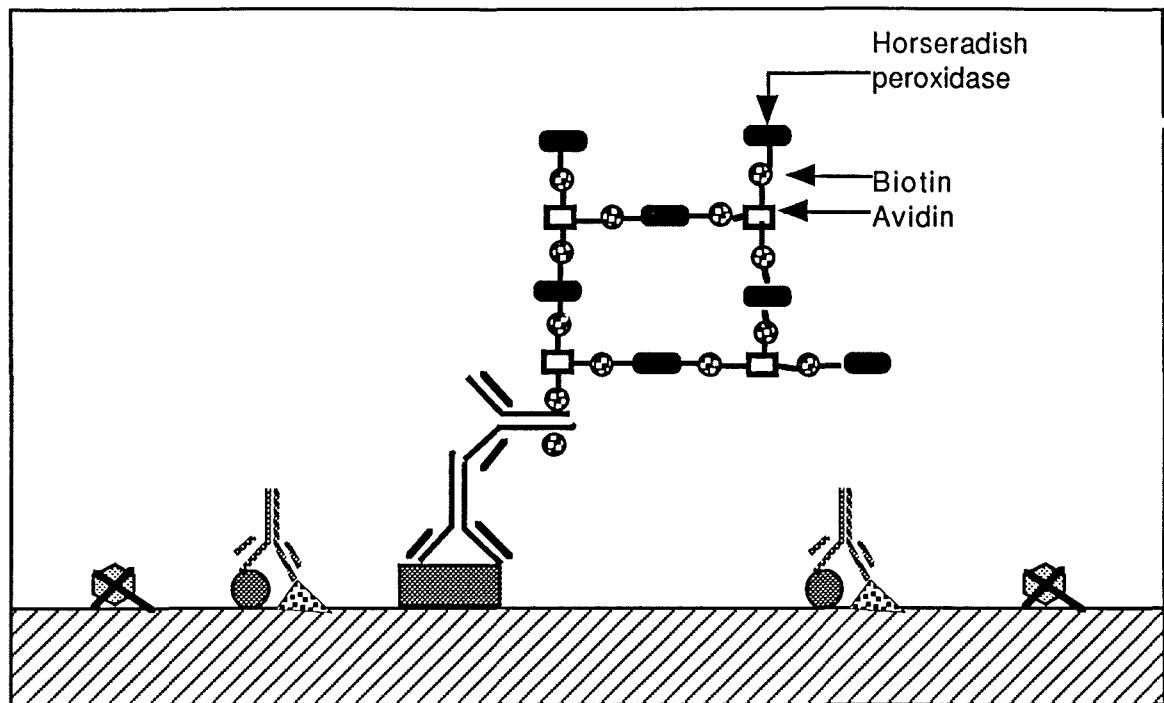


Figure 3.5. The preformed avidin-biotin horseradish peroxidase complex attaches to the biotinylated secondary antibody.

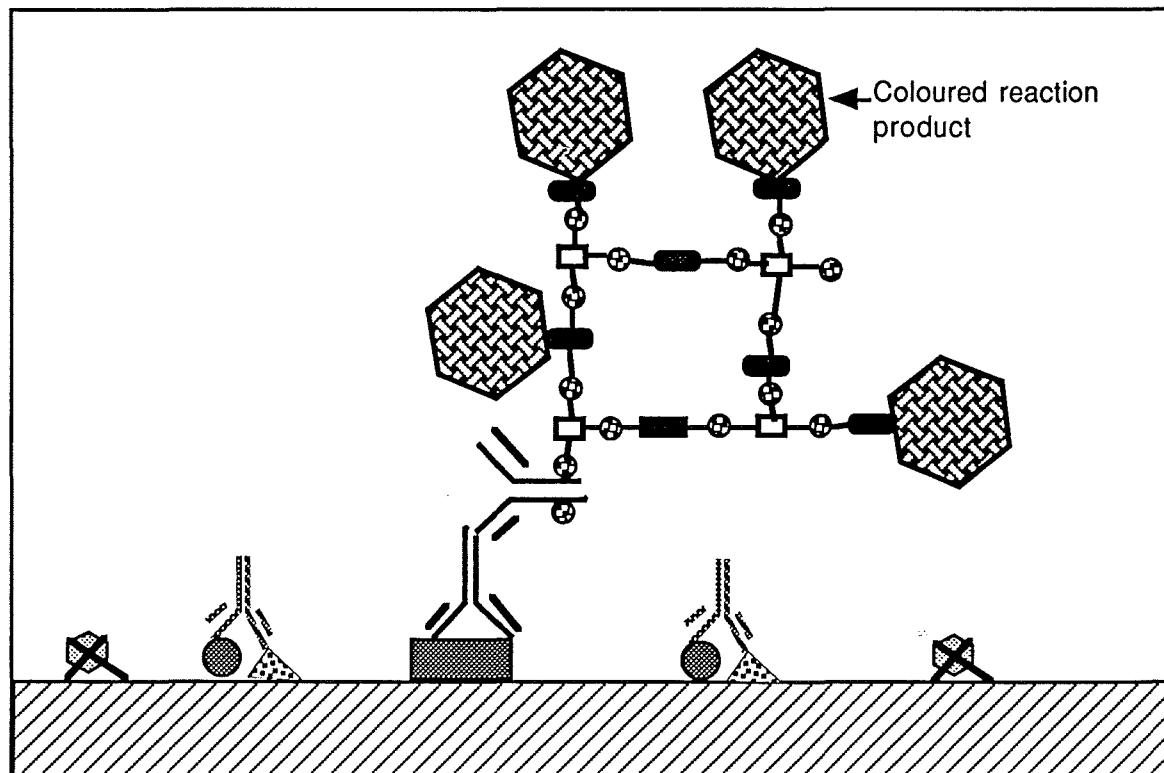


Figure 3.6. Addition of the chromogenic substrate (3,3-diaminobenzidine tetrahydrochloride) results in a coloured reaction product.

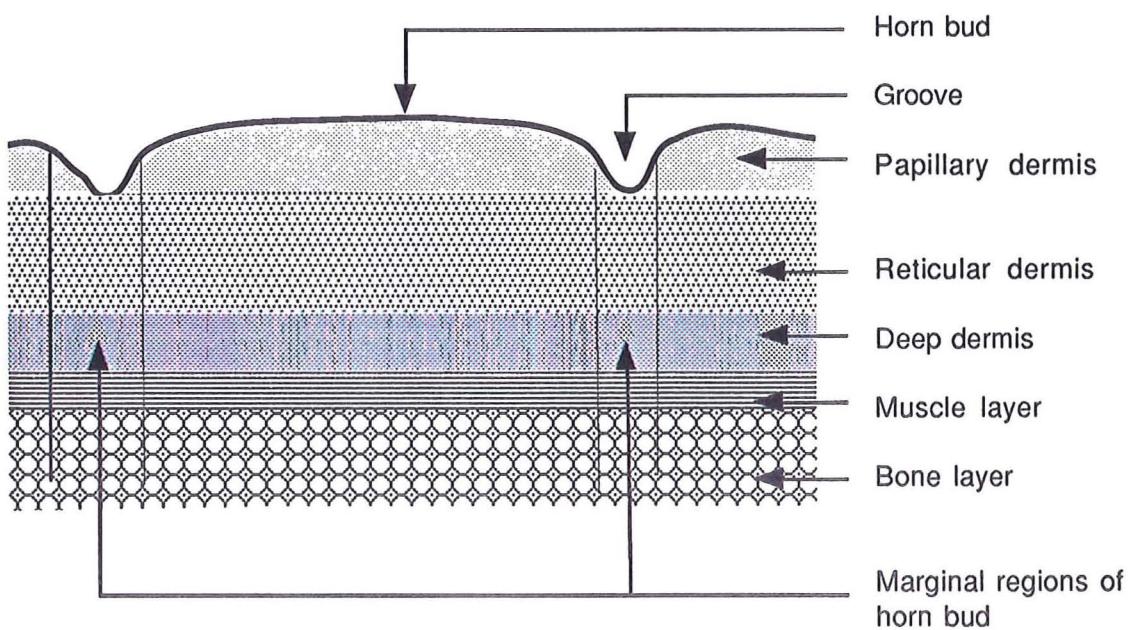


Figure 3.7 Schematic representation of horn bud and underlying layers.

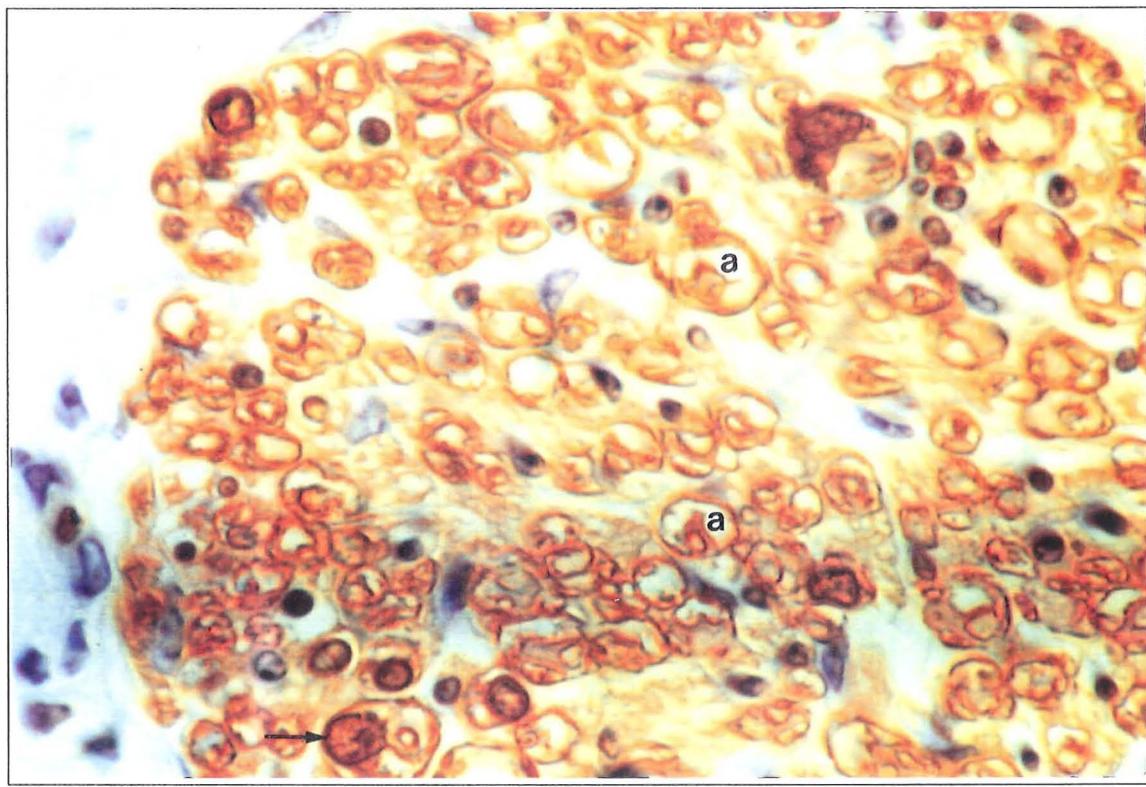


Figure 3.8. 4-week-old calf. Nerve fascicle in the deep dermis. Many of the nerve fibres are formed by S-100 immunoreactive cytoplasm enclosing irregular, crescent-shaped spaces (a). A few intensely S-100 immunoreactive Schwann cell nuclei (arrow) are present in this section. Anti-S-100. X 1,875.

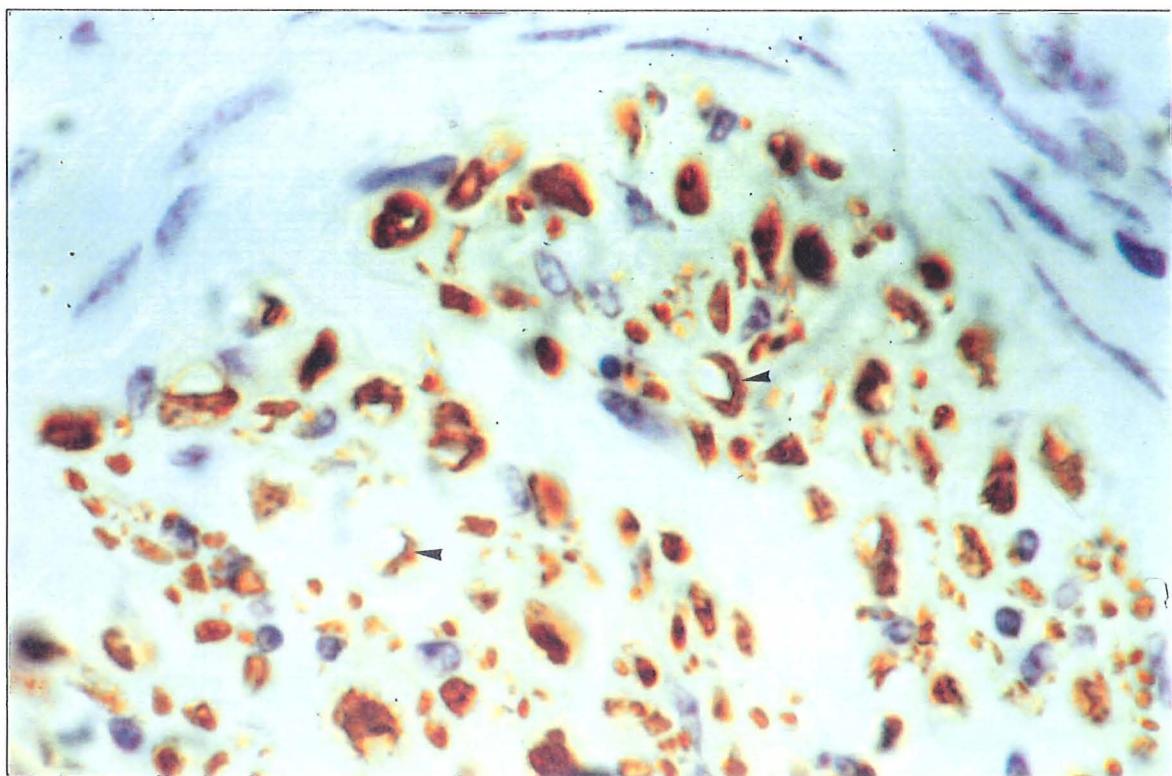


Figure 3.9. 4-week-old calf. Transverse section through a dermal nerve stained for neurofilament protein. Note the irregular axonal profiles (arrowheads). Anti-neurofilament. X 1,875.

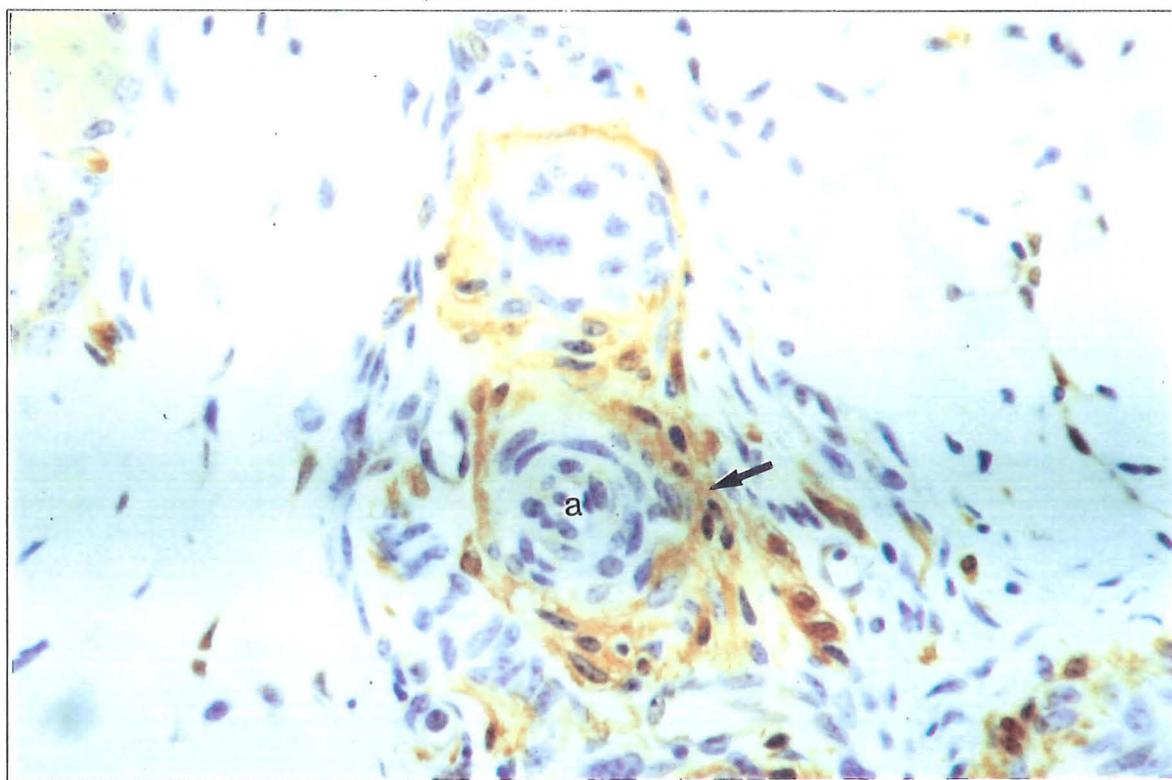


Figure 3.10. 1-week-old calf. S-100 immunoreactive nerve fibres (arrow) form concentric layers around arteriovenous anastomoses (a). The nerve fibres are restricted to the outer walls of the arteriovenous anastomoses with no nerves extending between the smooth muscle cells. Anti-S-100. X 750.

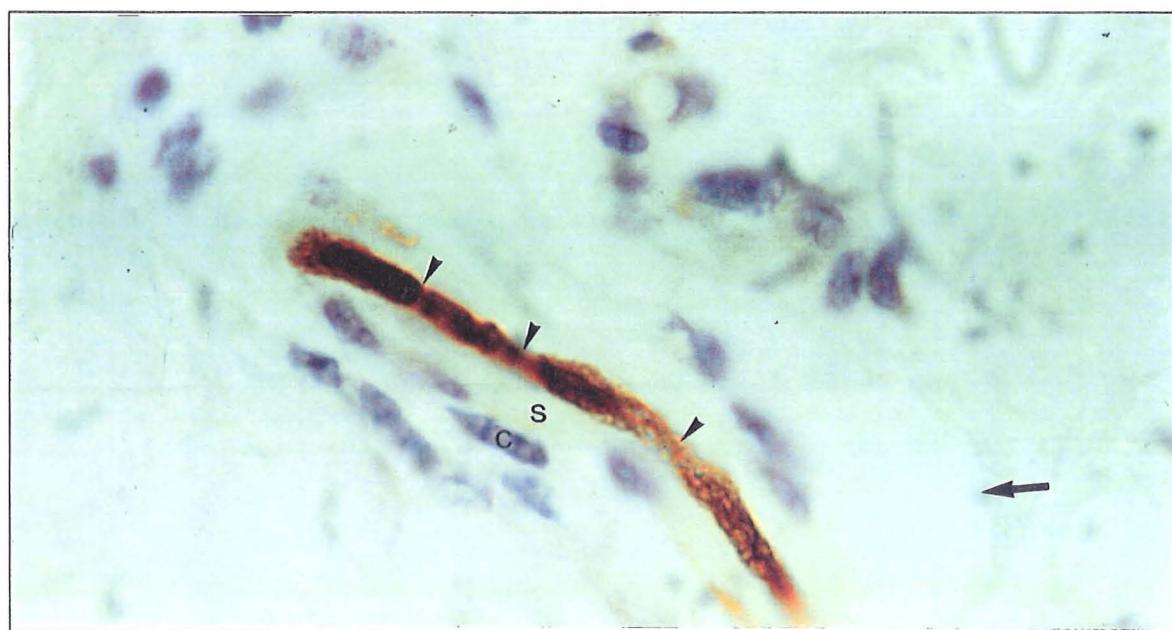


Figure 3.11. 2-day-old calf. A large bulbous corpuscle in the reticular dermis. The central neurofilament immunoreactive axon has several constrictions along its length (arrowheads). A faintly immunoreactive area (s) separates the axon from a layer of lamellar cells with elongated nuclei (c). A capsule (arrow) separates the corpuscle from the surrounding connective tissue. Anti-neurofilament. X 1,875.

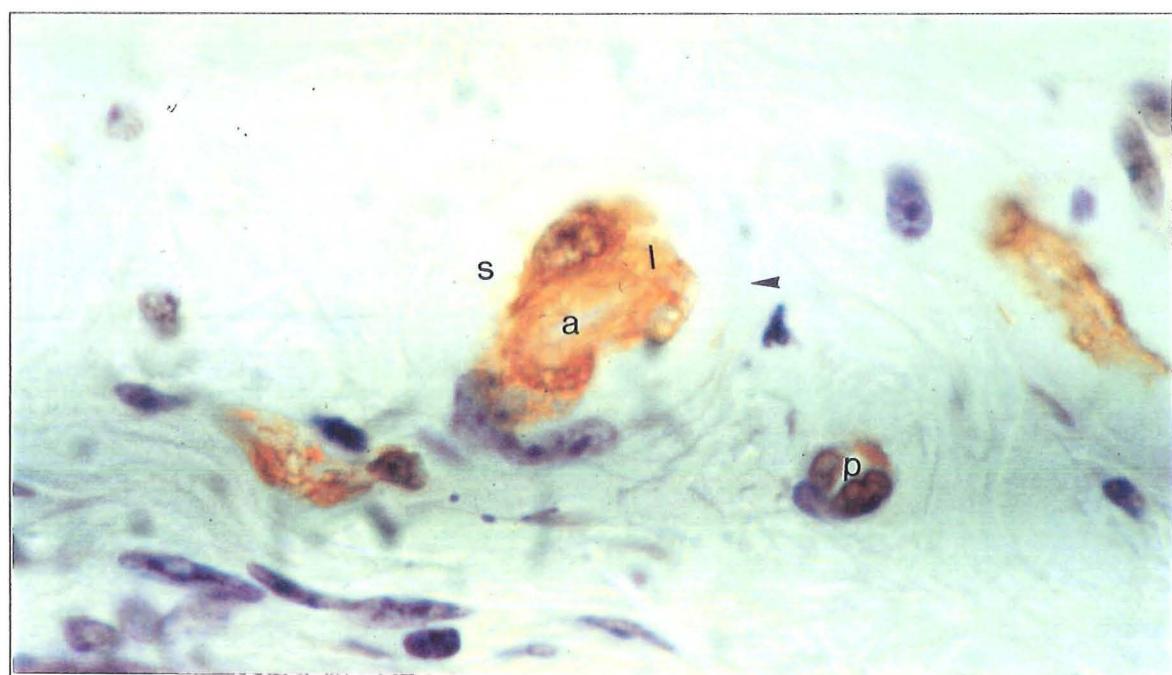


Figure 3.12. 1-week-old calf. Transverse section through a bulbous corpuscle. A S-100 immunoreactive inner core (l) encloses an elongated clear area (a) which is presumably the location of the axon. The inner core is formed by a mass of cytoplasmic processes from lamellar cells. A clear area (s) separates the inner core from the capsule (arrowhead). On the right is part of the pre-terminal axon (p) which is not enclosed in inner core lamellae. Anti-S-100. X 1,875.

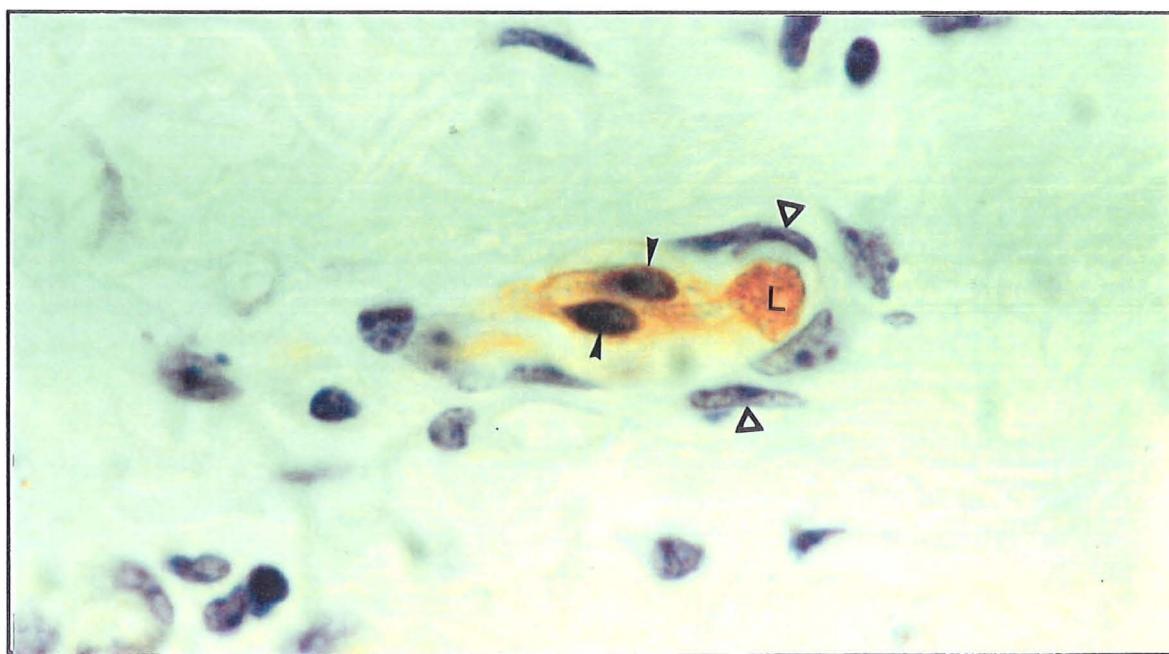


Figure 3.13. 1-week-old calf. A small bulbous corpuscle in the papillary dermis. Lamellar cells (small arrowheads) are seen surrounding the terminal axon, whilst the ultra-terminal segment is surrounded by a lamellar core (L). Cells with elongated nuclei surround the corpuscle (large arrowheads). Anti-S-100. X 1,875.

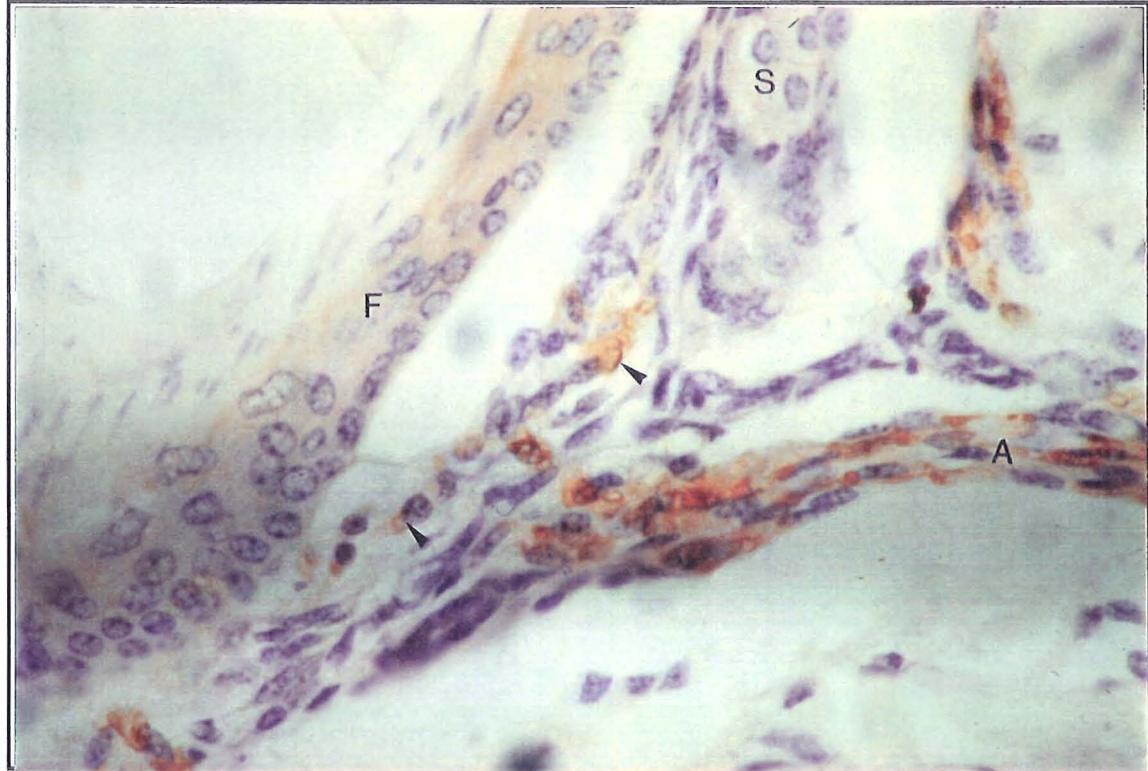


Figure 3.14 182-day-old bovine foetus. Transverse section of an hair follicle (F) and sebaceous gland (S) showing circularly-arranged nerve fibres (arrowheads) which branch from an afferent nerve (A). Anti-S-100. X 1,875.



Figure 3.15. 2-day-old calf. Longitudinal section of an hair follicle (F) and a sebaceous gland (S) at the site outlined in the line drawing. Three groups (G) of circularly orientated Ruffini terminals are seen between the hair follicle and sebaceous gland. Each group of Ruffini terminals consists of several Schwann cells (arrow) with clear spaces indicating the location of axons. Separating the groups of Ruffini terminals are fibrocyte-like septal cells (arrowhead). Anti-S-100. X 750.

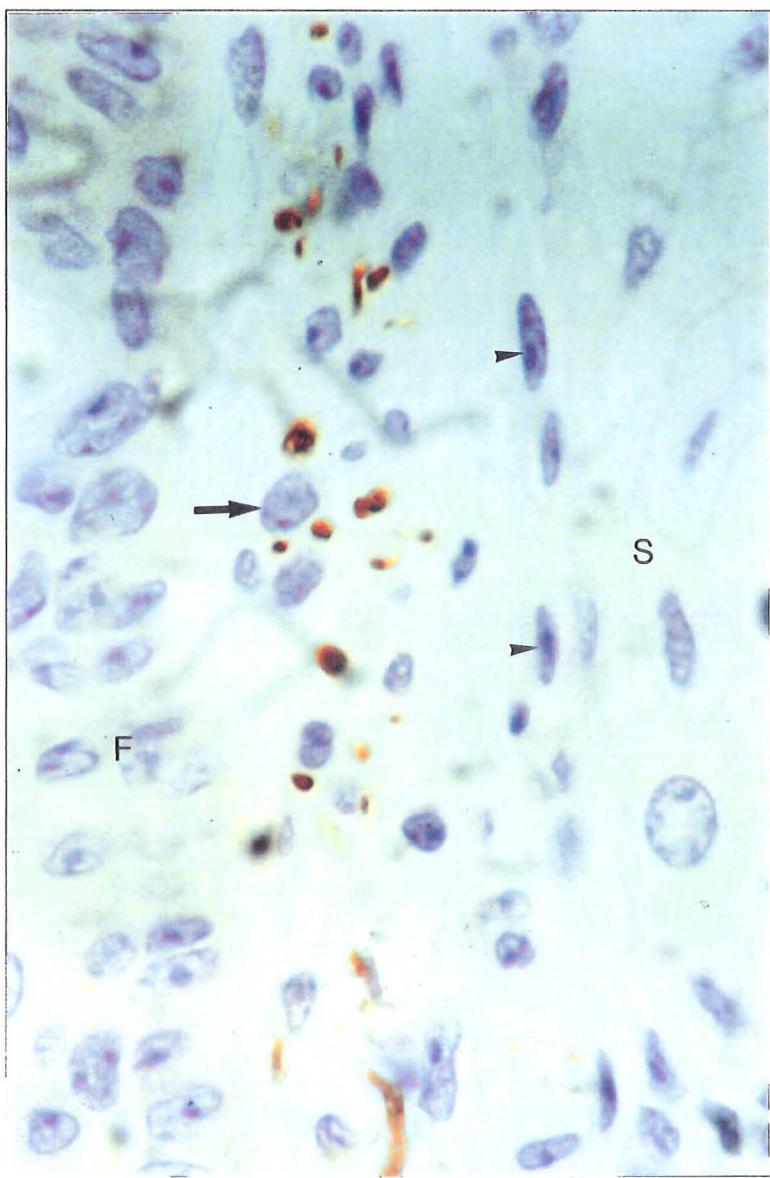


Figure 3.16. 2-day-old calf. Longitudinal section of an hair follicle (F) and sebaceous gland (S) showing neurofilament-immunoreactive Ruffini terminals. A few of the axons are close to round, Schwann cell nuclei (arrow). A layer of fibrocytes (arrowheads) marks the outer-limit of the connective tissue sheath surrounding the hair follicle. Anti-neurofilament. X 750.

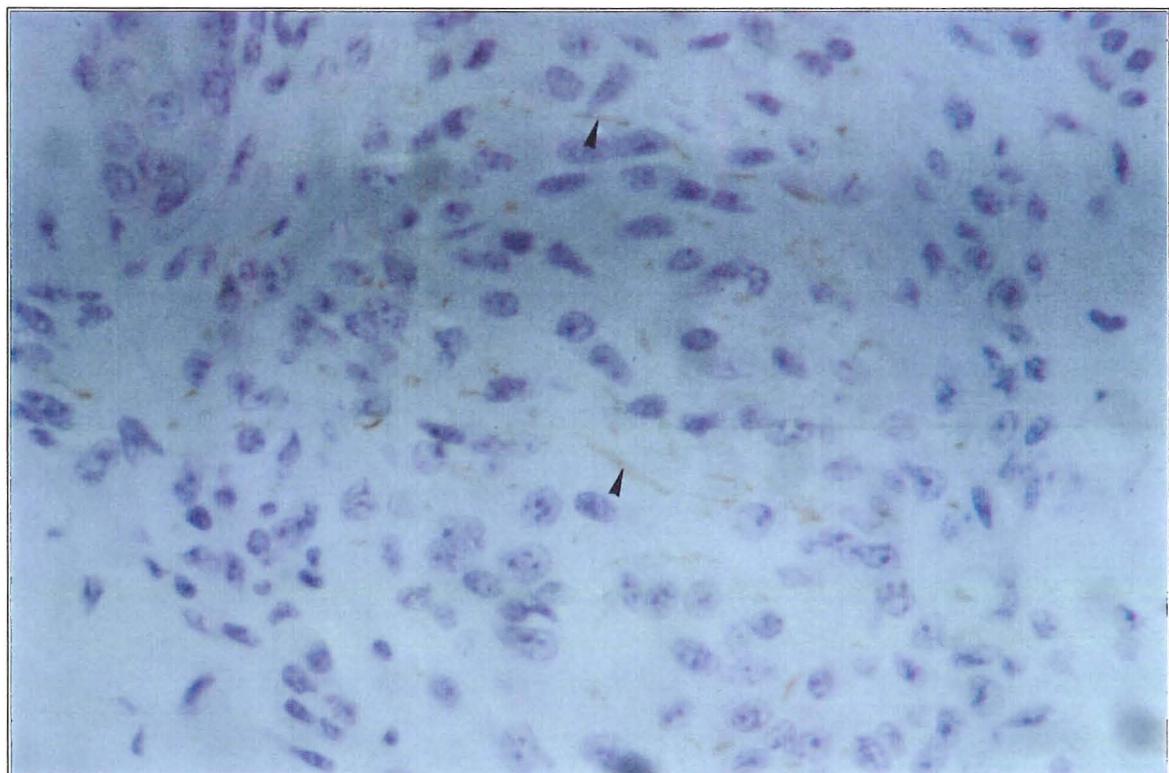


Figure 3.17. 4-week-old calf. In the advanced stages of hair follicle degeneration groups of cells with a few nerve fibres (arrowheads) are present. Anti-neurofilament. X 750.

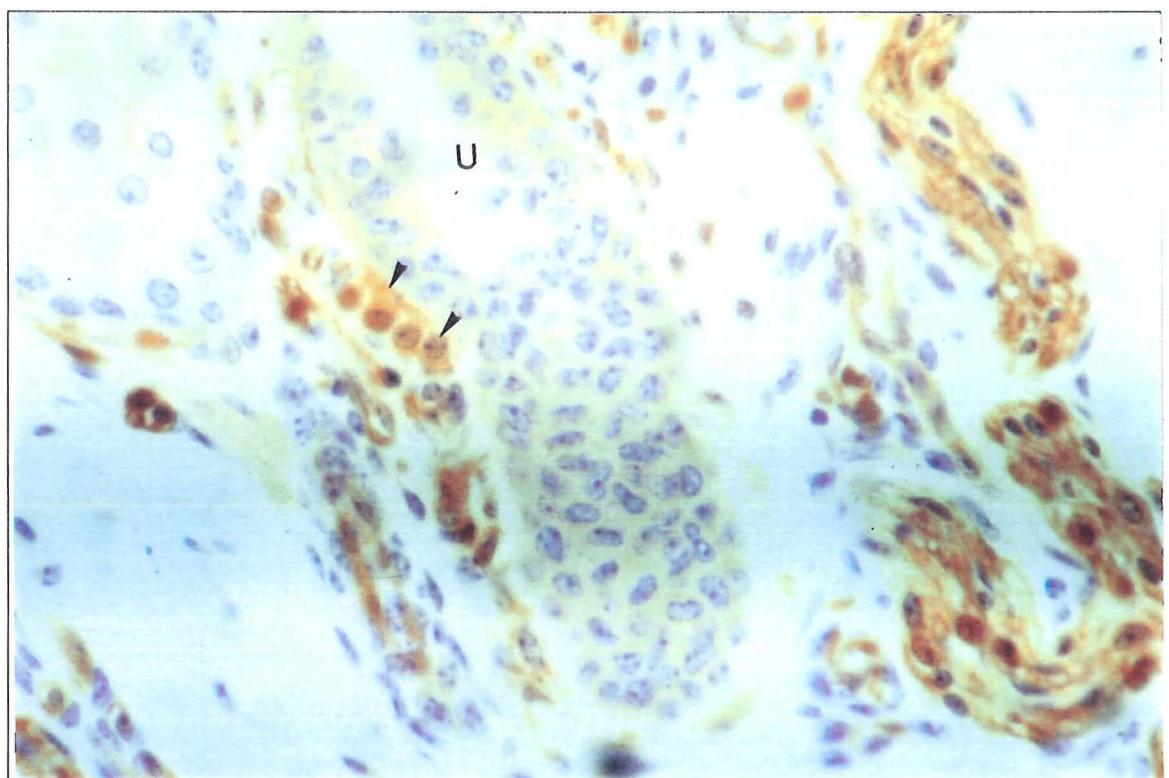


Figure 3.18. 2-day-old calf. The upper part of the hair follicle (U) has been cut obliquely revealing the palisade arrangement of perifollicular terminals (arrowheads). Anti-S-100. X 750.



Figure 3.19 2-day-old calf. Longitudinal section through an hair follicle (F) and sebaceous gland (S). Several perifollicular nerves (small arrowheads) orientated parallel to the hair follicle are seen. Circularly arranged nerves are observed between the hair follicle and sebaceous gland (large arrowheads). Anti-neurofilament. X 750.

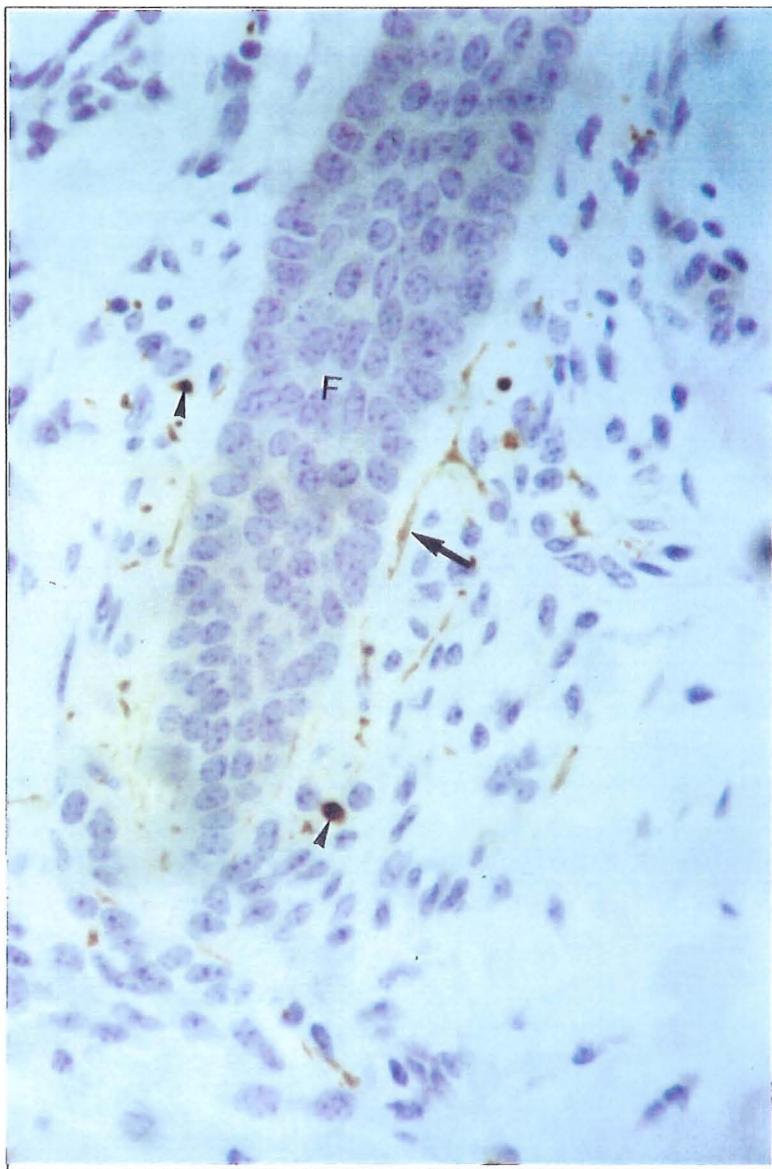


Figure 3.20. 2-week-old calf. Intact neurofilament-immunoreactive axons are seen around a longitudinally sectioned degenerating hair follicle. Longitudinally orientated nerves (arrow) are placed closest to the hair follicle (F). Transverse profiles of circularly arranged nerves (arrowheads) are seen amongst the cells surrounding the hair follicle. Anti-neurofilament. X 750.

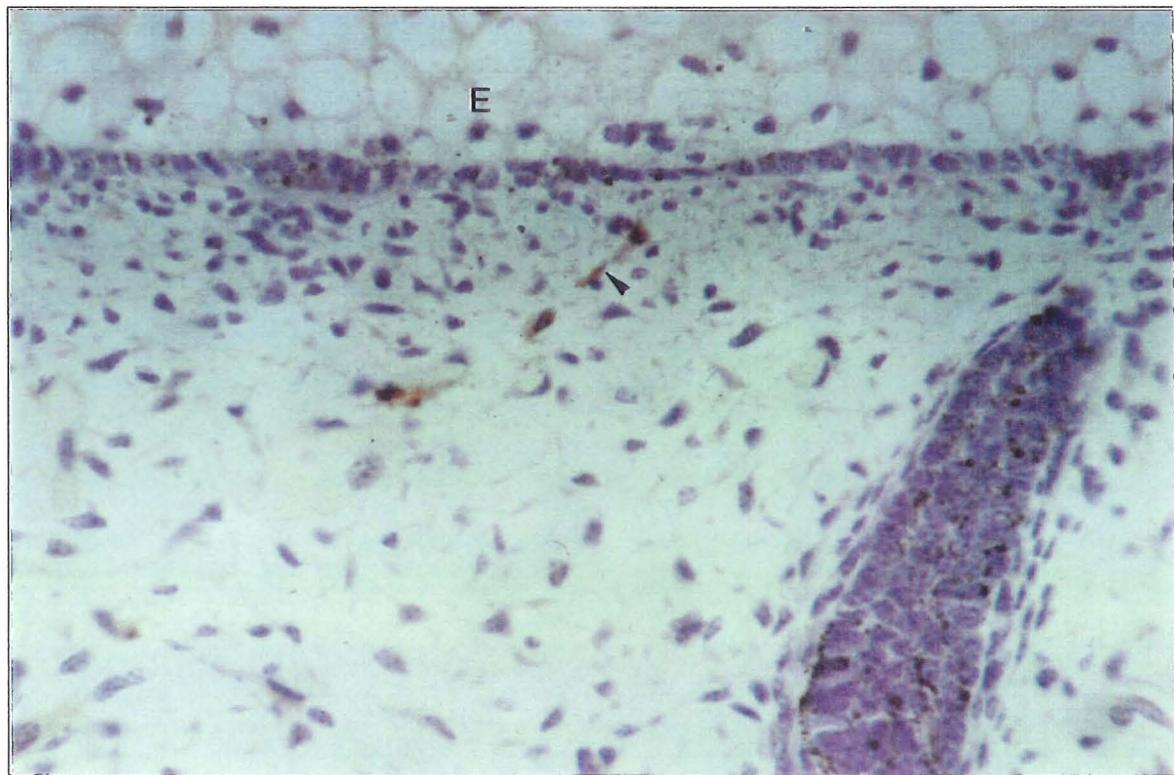


Figure 3.21. 112-day-old bovine foetus. A free nerve ending (arrowhead) unaccompanied by perineurial cells courses towards the epidermis (E). Anti-S-100. X 750.

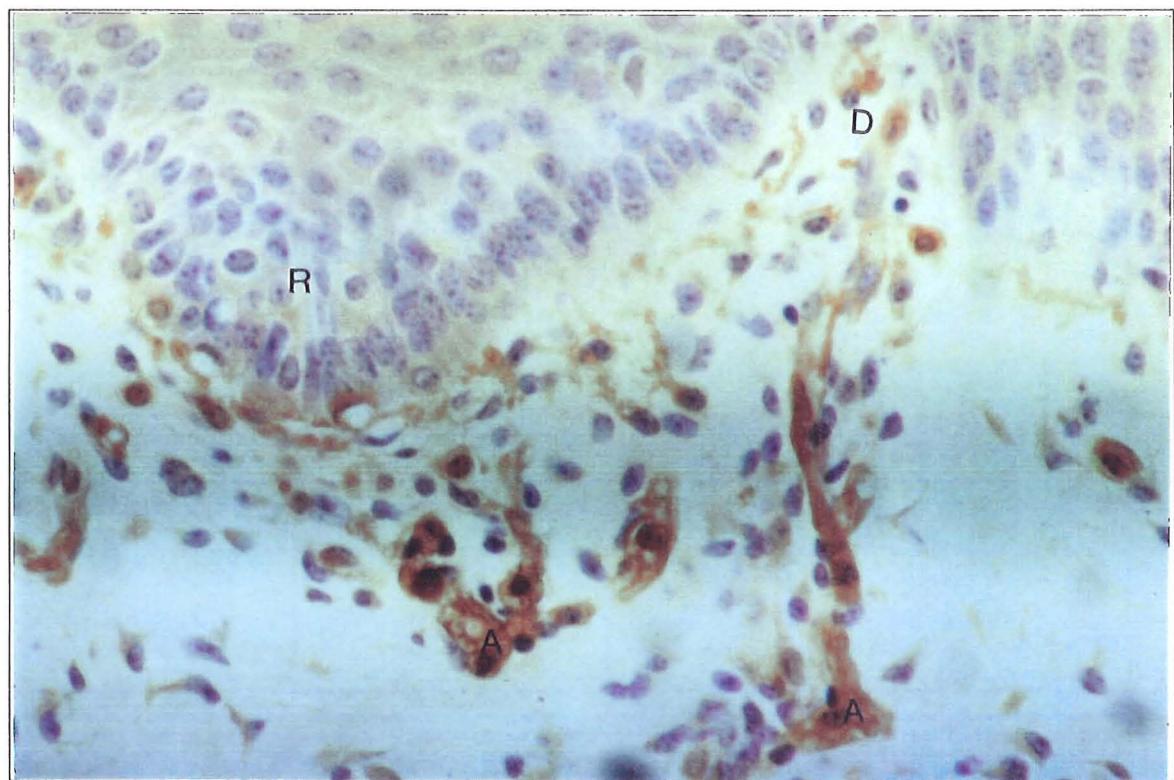


Figure 3.22. 3-week-old calf. Afferent nerves (A) divide extensively to form a terminal tree of free nerve endings, which supply an epidermal ridge (R). Several free nerve endings extend into adjacent dermal papillae (D). Anti-S-100. X 750

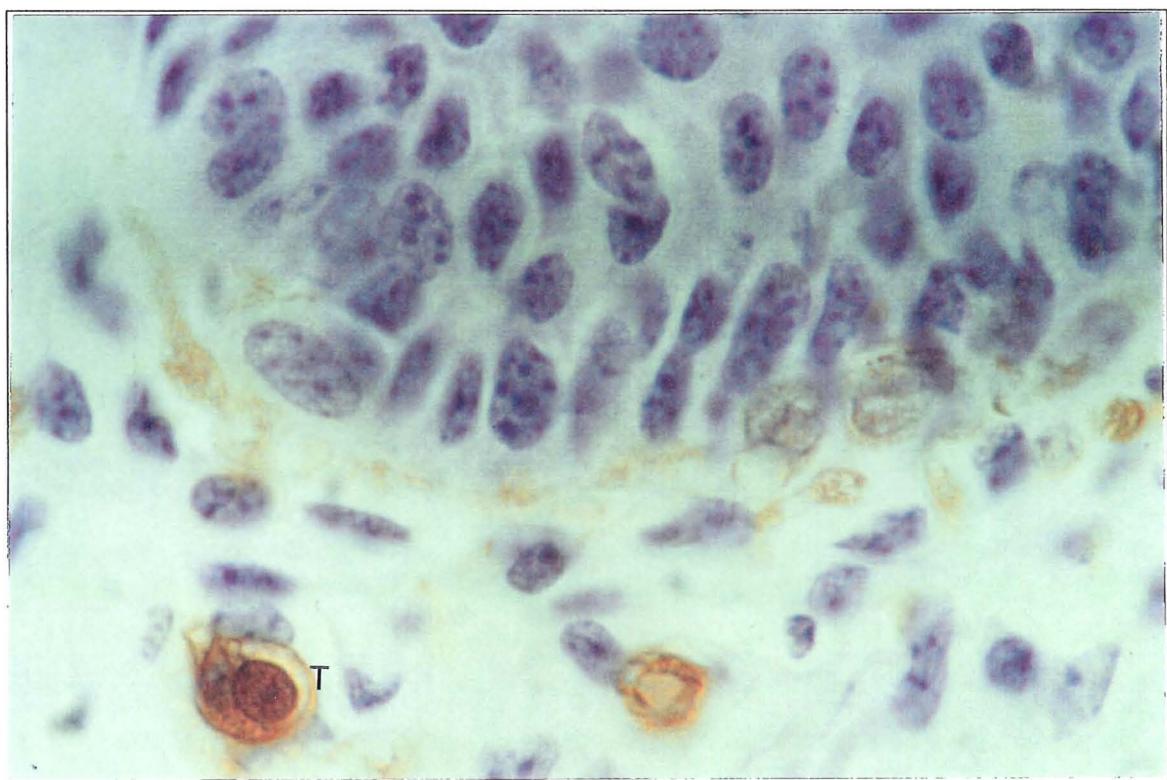


Figure 3.23. 4-week-old calf. A terminal Schwann cell (T) is seen below an epidermal ridge. The cytoplasm at one end of the cell is intensely immunoreactive. Anti-S-100. X 1,875.

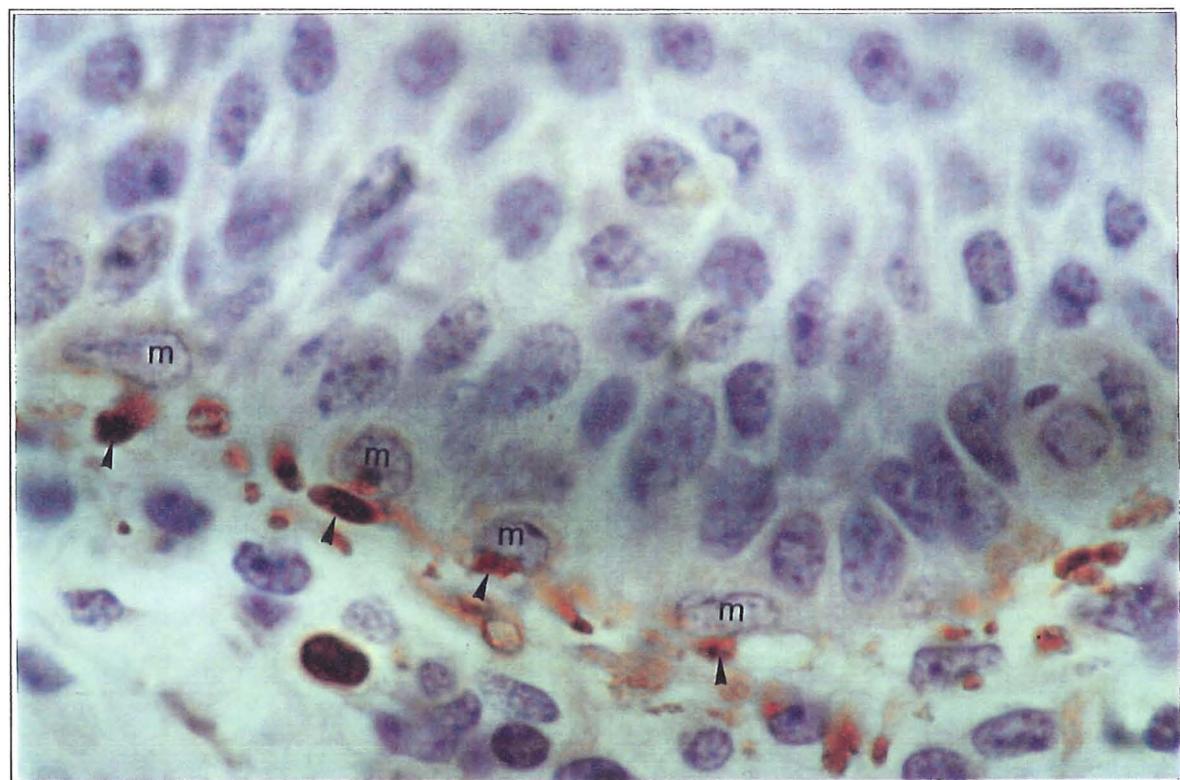


Figure 3.24. 2-day-old calf. Anti-neurofilament sera demonstrates thickened nerve terminals (arrowheads) contacting Merkel cells (m). In most cases the nerve endings appear to invaginate the cells close to the nuclei. Anti-neurofilament. X 1,875.

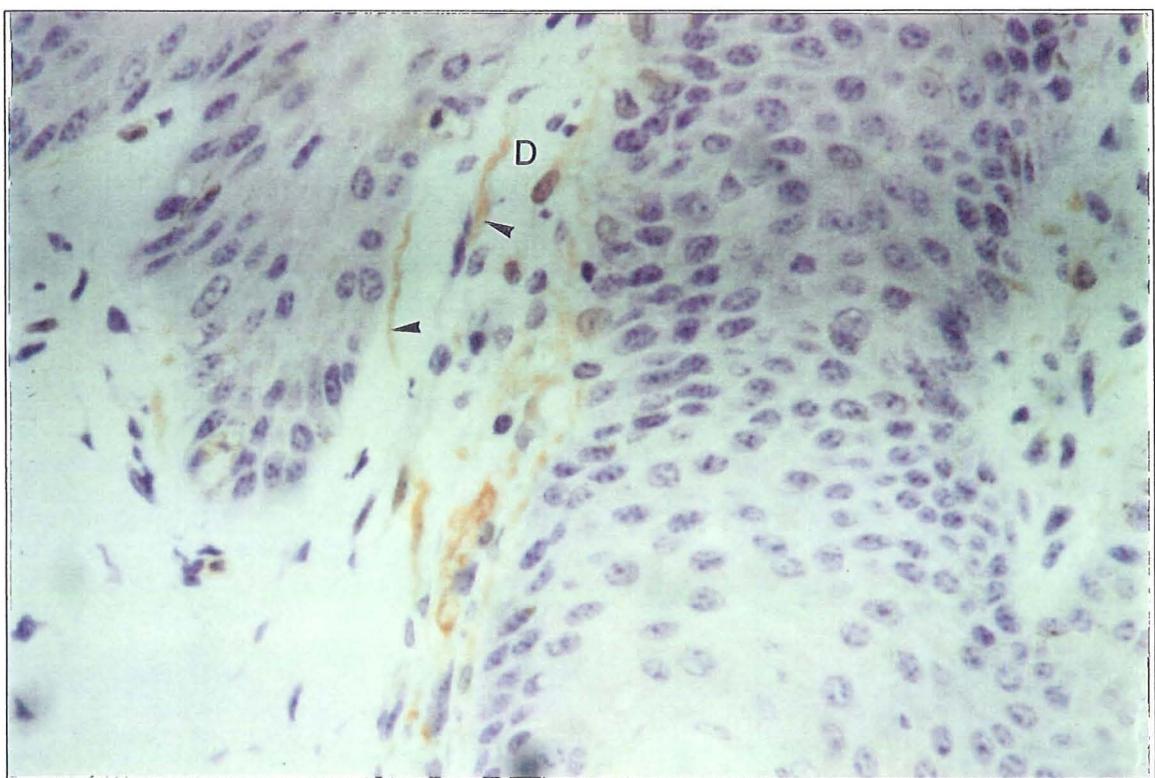


Figure 3.25 4-week-old calf. A few straight, unbranched free nerve endings (arrowheads) enter a dermal papilla (D). Anti-S-100. X 750.

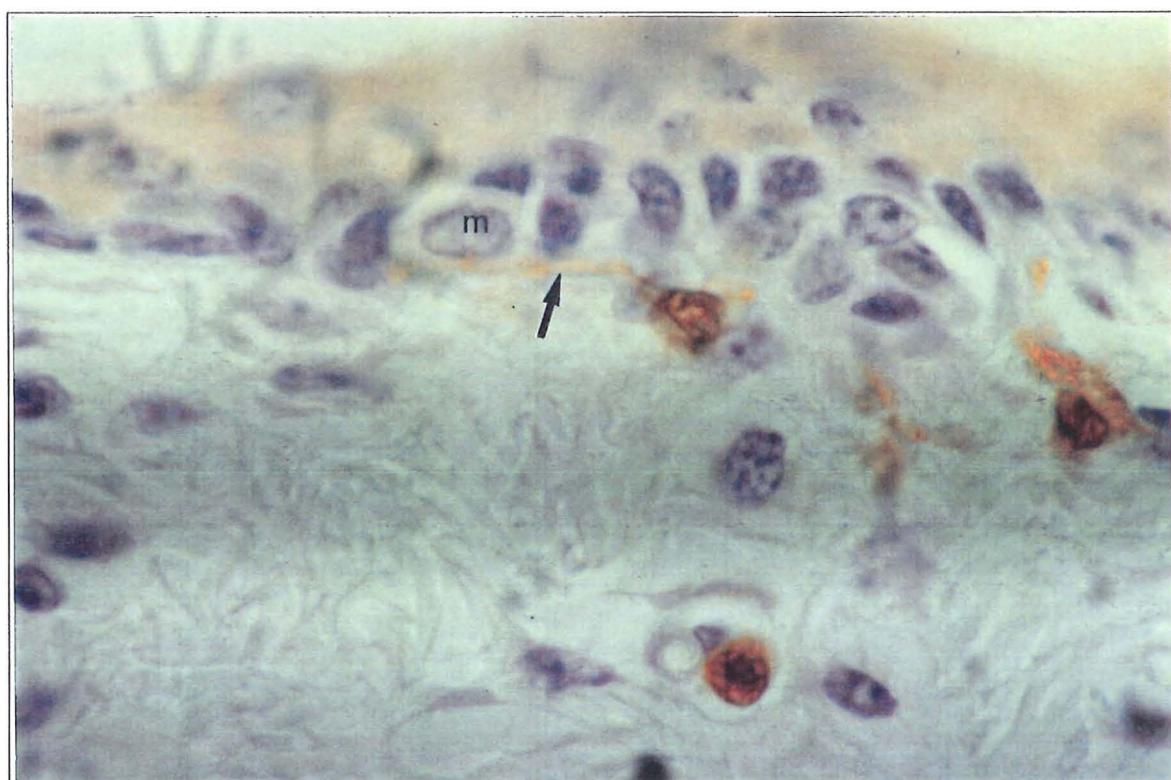


Figure 3.26 182-day-old bovine foetus. Isolated Merkel cell (m) associated with a nerve fibre (arrow). Note the halo of clear cytoplasm surrounding the Merkel cell nucleus. Anti-S-100. X 1,875.

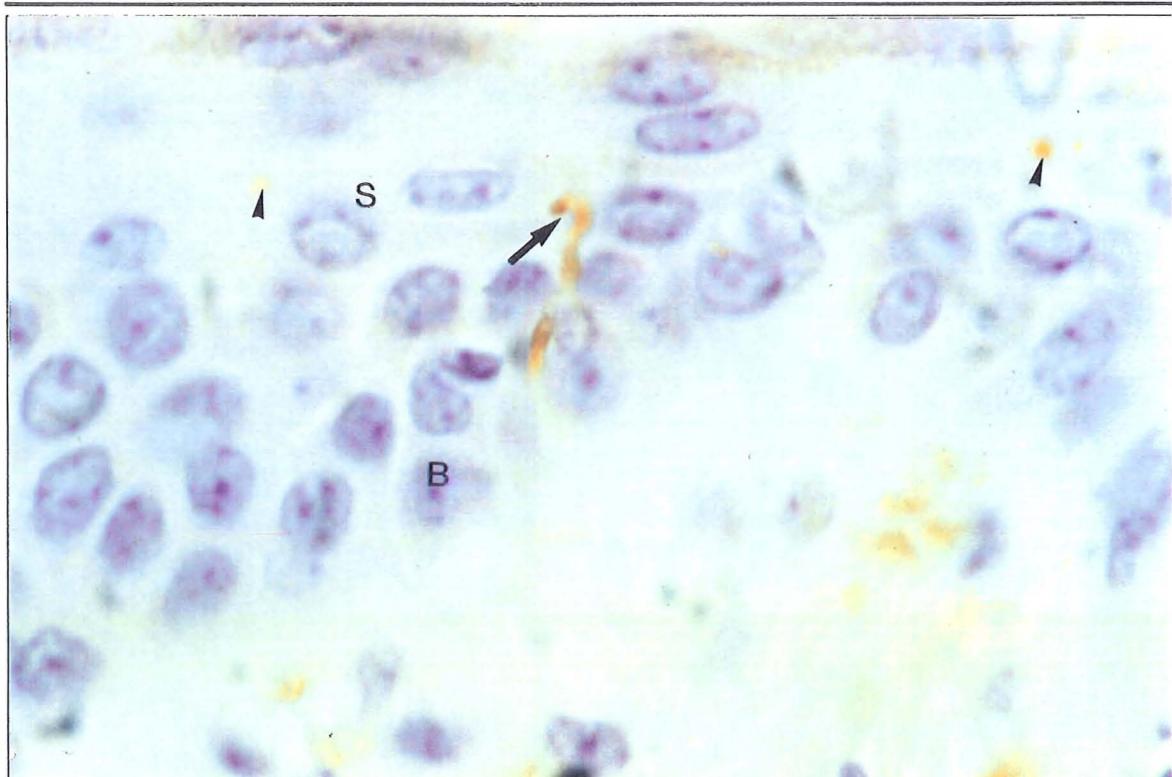


Figure 3.27 2-day-old calf. A Schwann cell process extends between the cells of the stratum basale (B) and stratum spinosum (S). The cytoplasmic process ends in the stratum spinosum in a curved ending (arrow). Two other Schwann cell profiles are present in the epidermis (arrowheads). Anti-S-100. X 1,875.

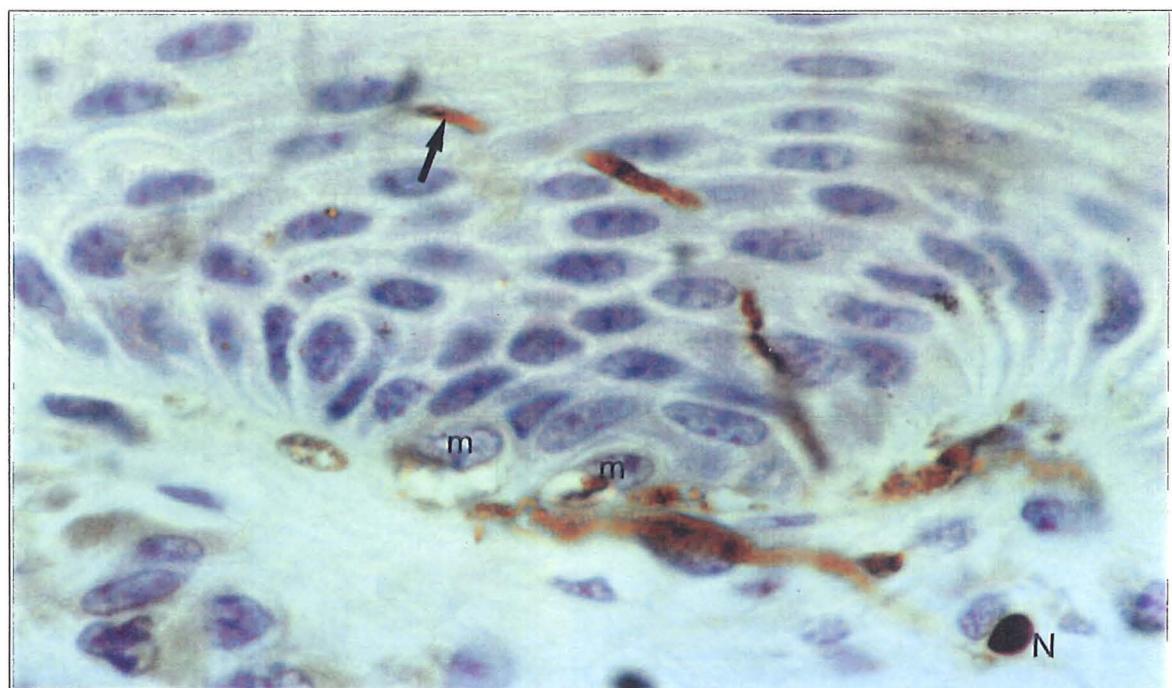


Figure 3.28 2-day-old calf. Free nerve endings branch from a subepidermal nerve (N). One of the branches enters the epidermis, courses through the stratum spinosum and terminates in a tapered ending (arrow). The other nerve maintains a subepidermal position ending on Merkel cells (m). Anti-neurofilament. X 1,875.

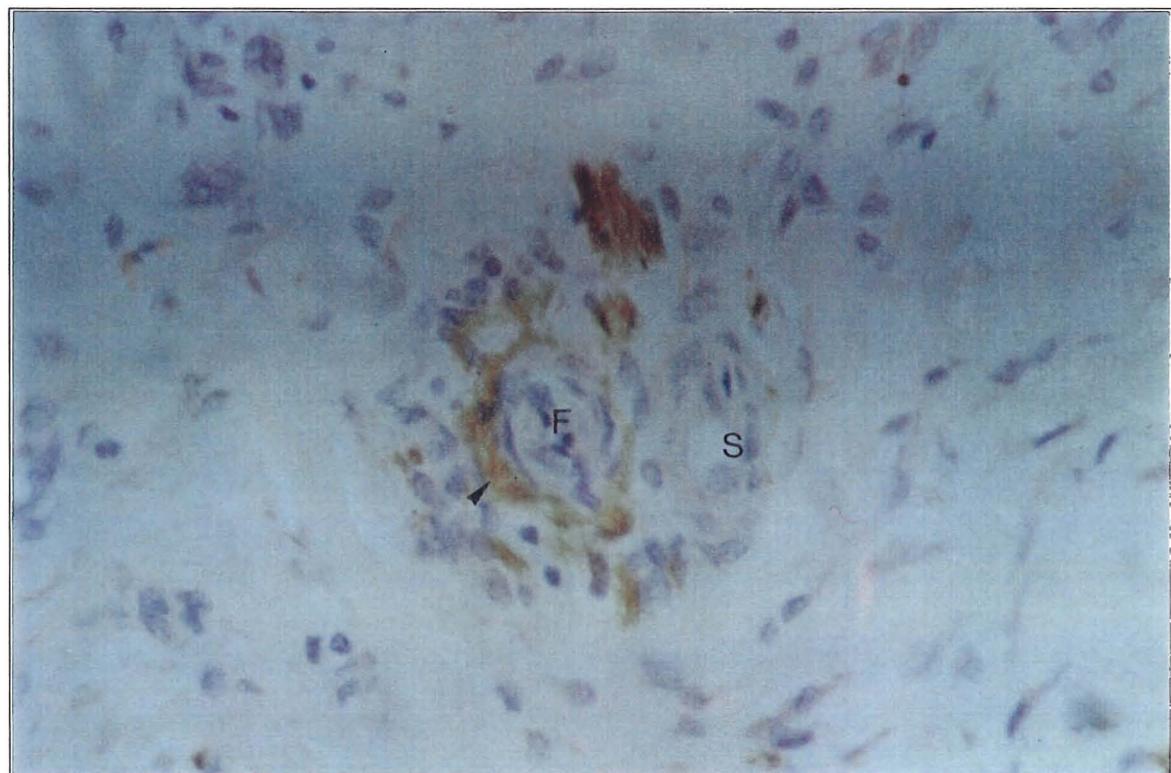


Figure 3.29. 2-week-old kid. Transverse section through a degenerating hair follicle (F) and sebaceous gland (S). The hair follicle is surrounded by S-100 immunoreactive nerve fibres (arrowhead). Anti-S-100. X 750.

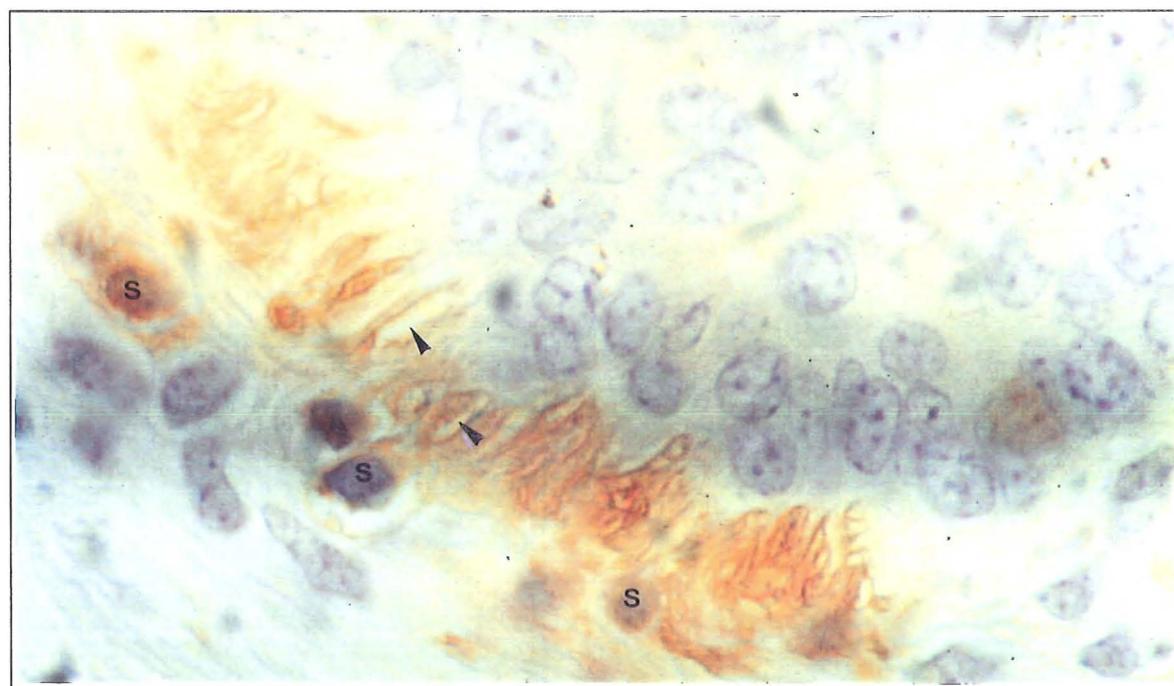
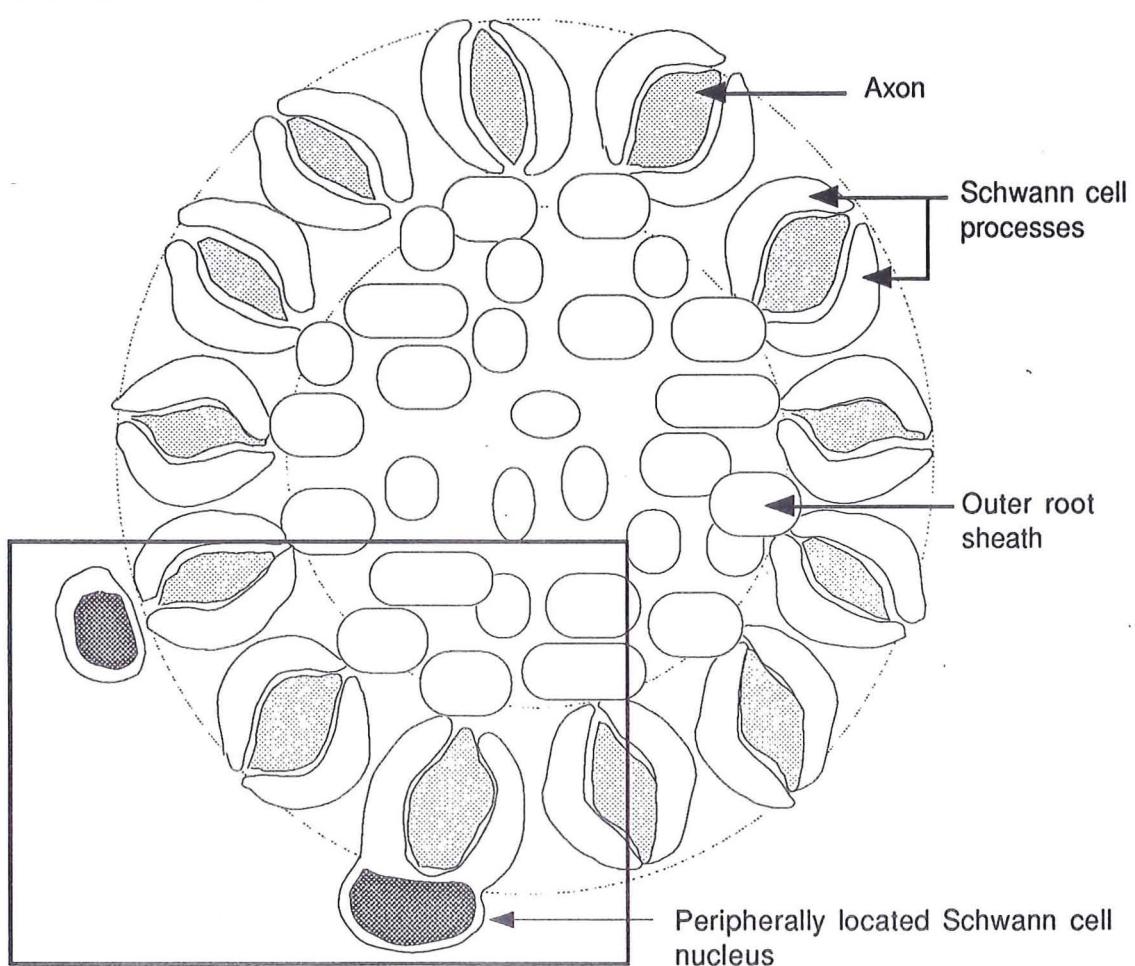


Figure 3.30. 2-week-old kid. Transverse section through part of a hair follicle in the area shown in the line drawing. Immunoreactive cytoplasmic processes enclose elongated spaces (arrowheads) which are the presumptive locations of axons. Note the presence of peripherally located Schwann cell nuclei (s). Anti-S-100. X 1,875.

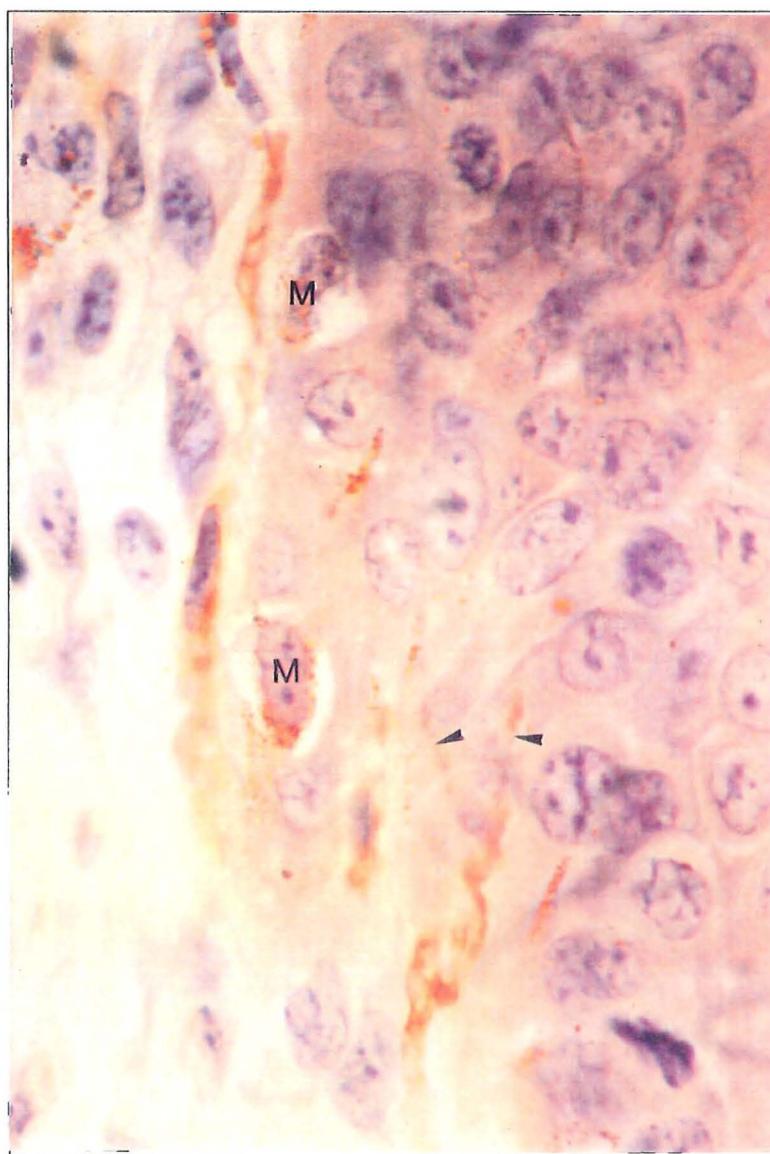


Figure 3.31 10-hour-old kid. Free nerve endings course through a dermal papilla in close contact to the stratum basale and Merkel cells (M). A few of the nerves branch within the epidermis (arrowheads). Anti-S-100. X 1,875.

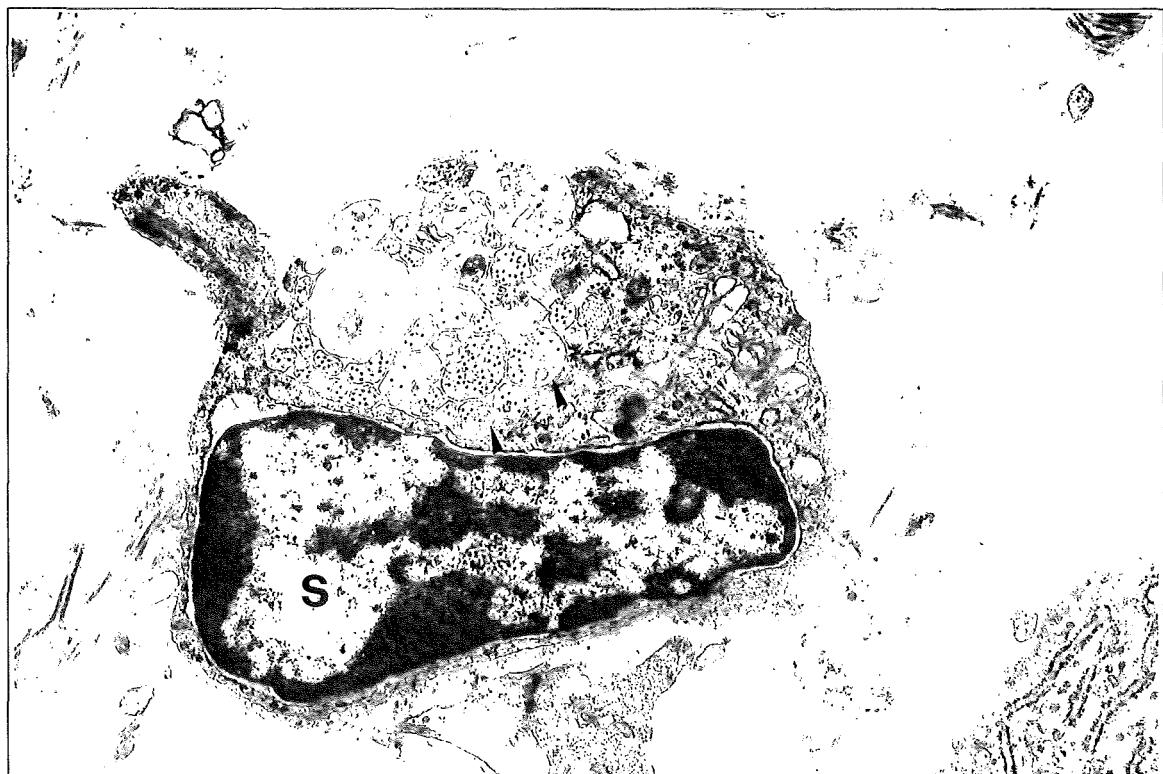


Figure 3.32. 112-day-old bovine foetus. Cross-section through an unmyelinated nerve. Axons (arrowheads) of various sizes invaginate the Schwann cell (S). TEM. X 5,400.

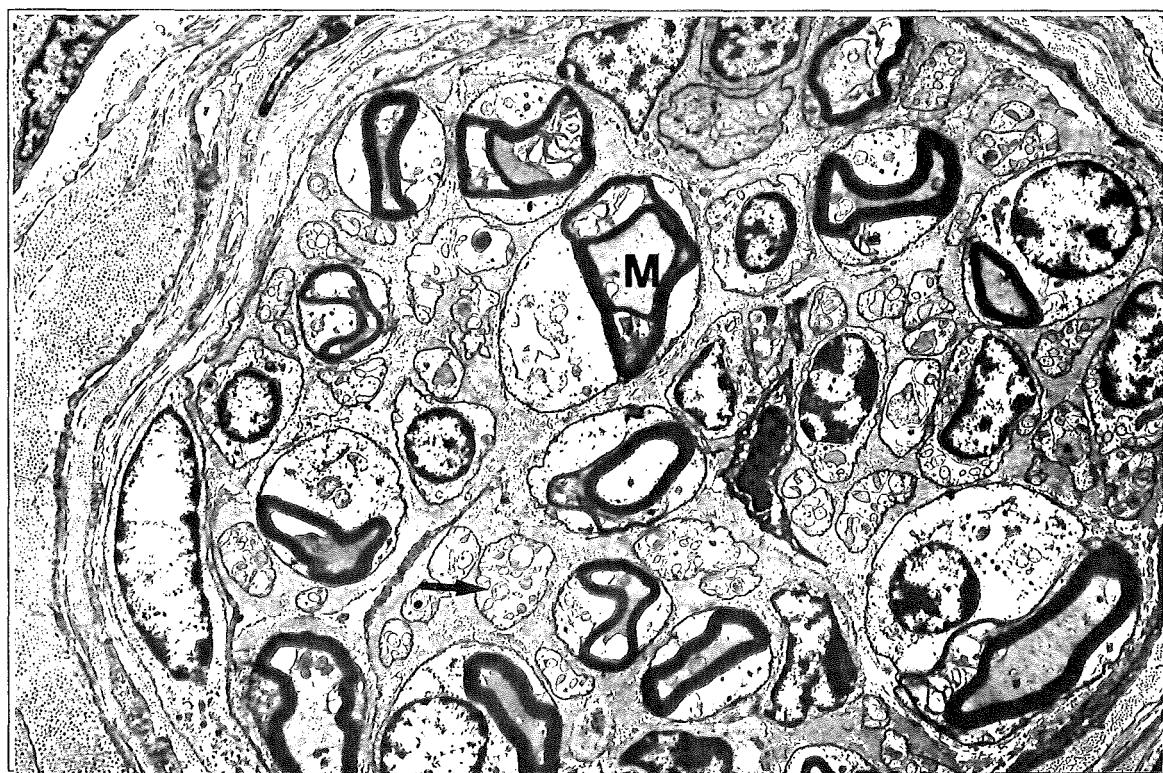


Figure 3.33. 4-week-old calf. A cross-sectional view of a nerve fascicle containing a mixture of myelinated (M) and unmyelinated (arrow) nerve fibres. TEM. X 4,000.

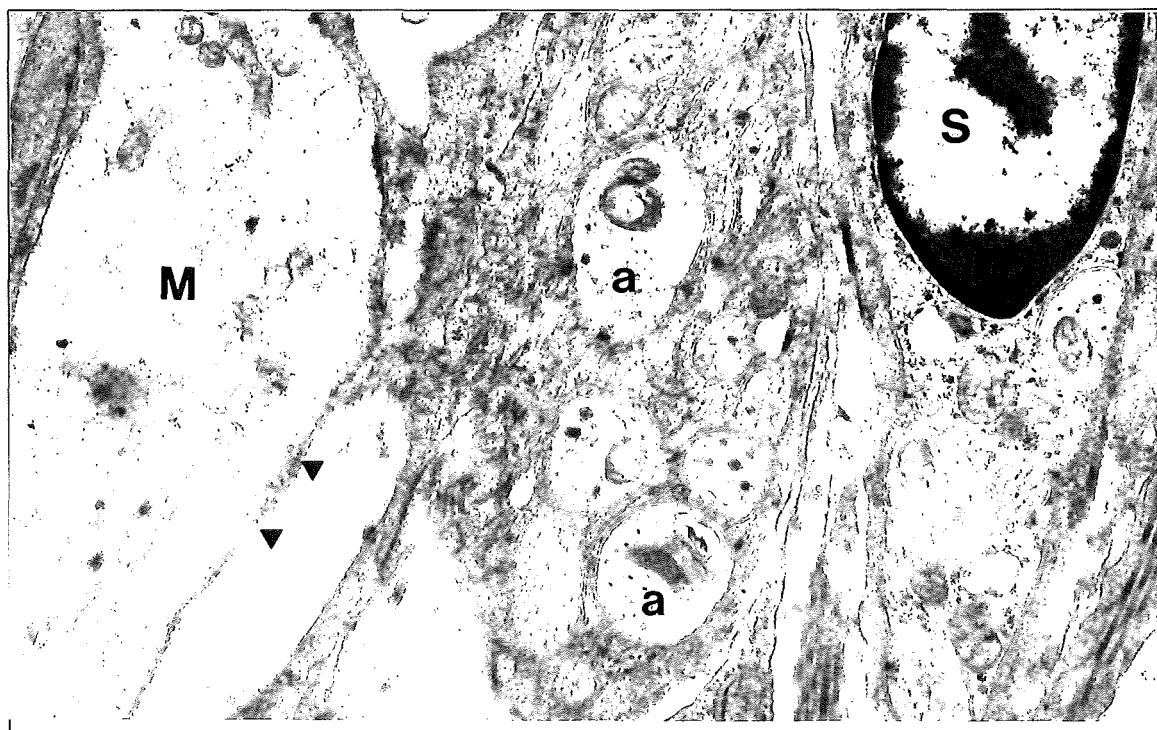


Figure 3.34. 4-week-old calf. Cross- section through the outer wall of an arteriovenous anastomosis. To the left is the wall of the arteriovenous anastomosis which is composed of smooth muscle cells (M). The smooth muscle cell is characterized by the presence of caveolae (arrowheads) and dense bodies along the sacrolemma. On the right is a Schwann cell nucleus (S). Several axons (a) containing vesicles and electron-dense bodies invaginate the Schwann cell cytoplasm. TEM. X 13,400.

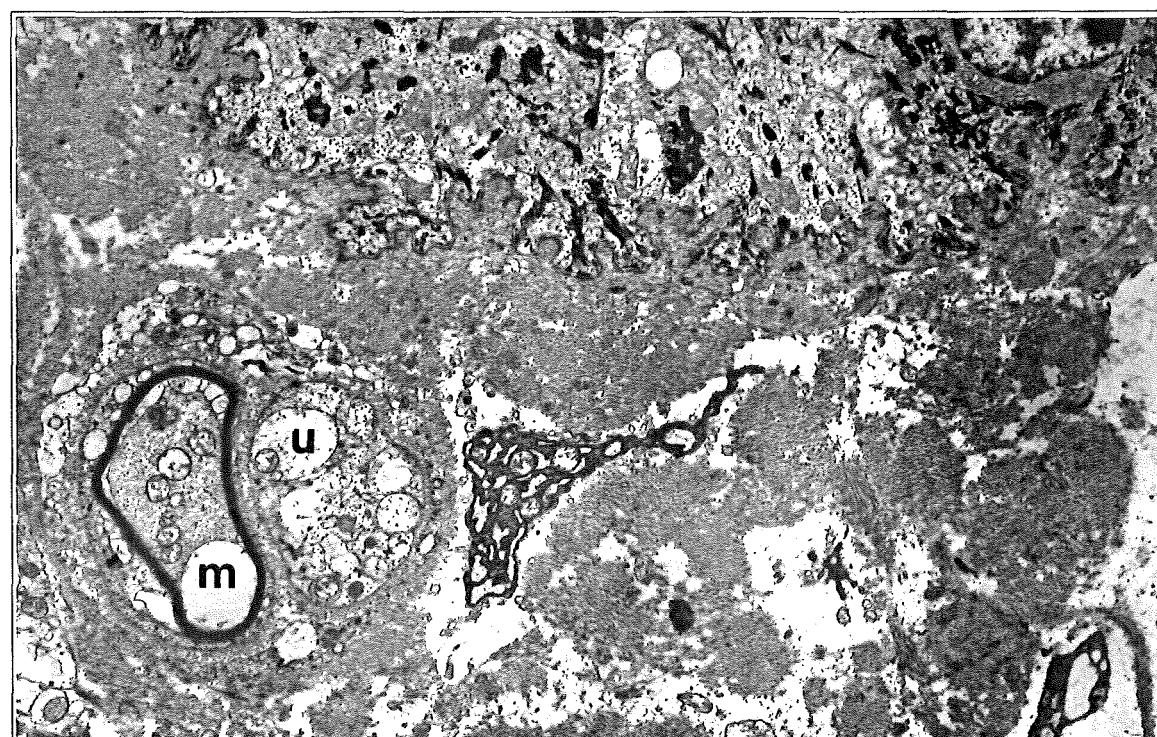


Figure 3.35. 3-week-old calf. Sub-epidermal nerves in the papillary dermis commonly contain myelinated (m) and unmyelinated (u) nerve fibres. TEM. X 5,400.

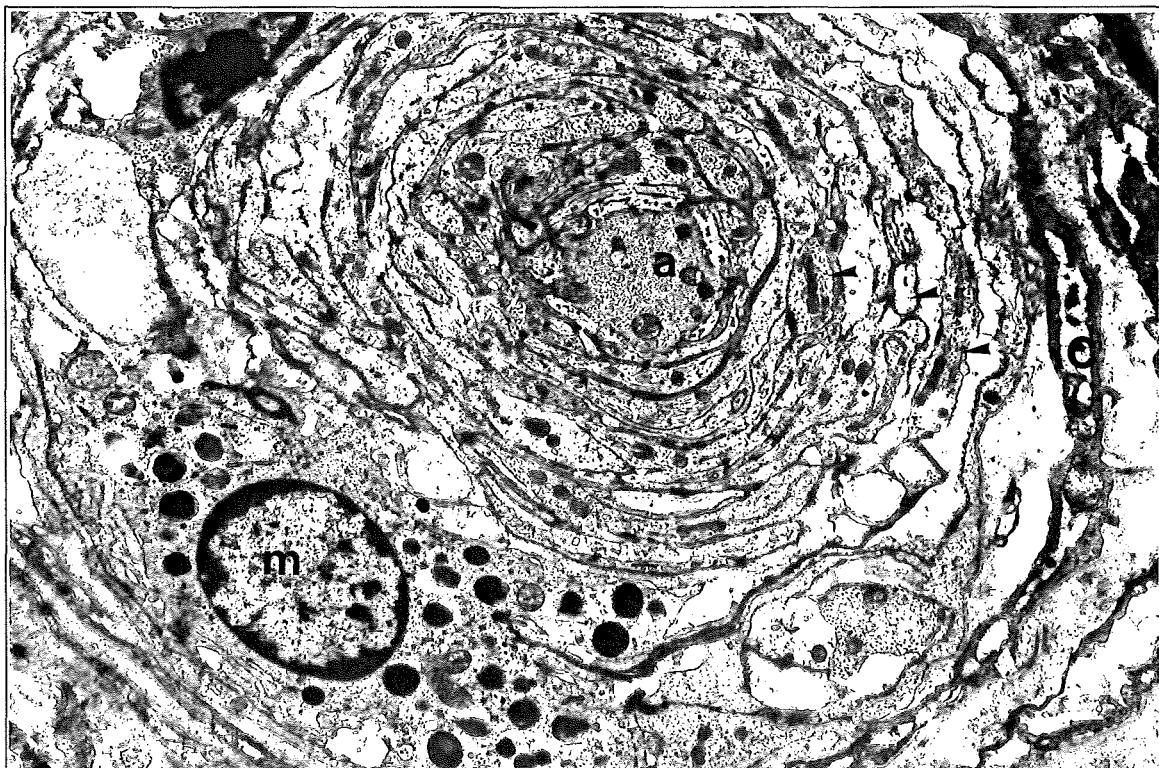


Figure 3.36. 4-week-old calf. Cross-section through the ultra-terminal segment of a bulbous corpuscle shows several concentric lamellae (arrow heads) around an axon (a). Note the peripheral arrangement of the mitochondria within the axon. A mast-like cell (m) is seen within the confines of the corpuscular capsule (C). TEM. X 8,000.

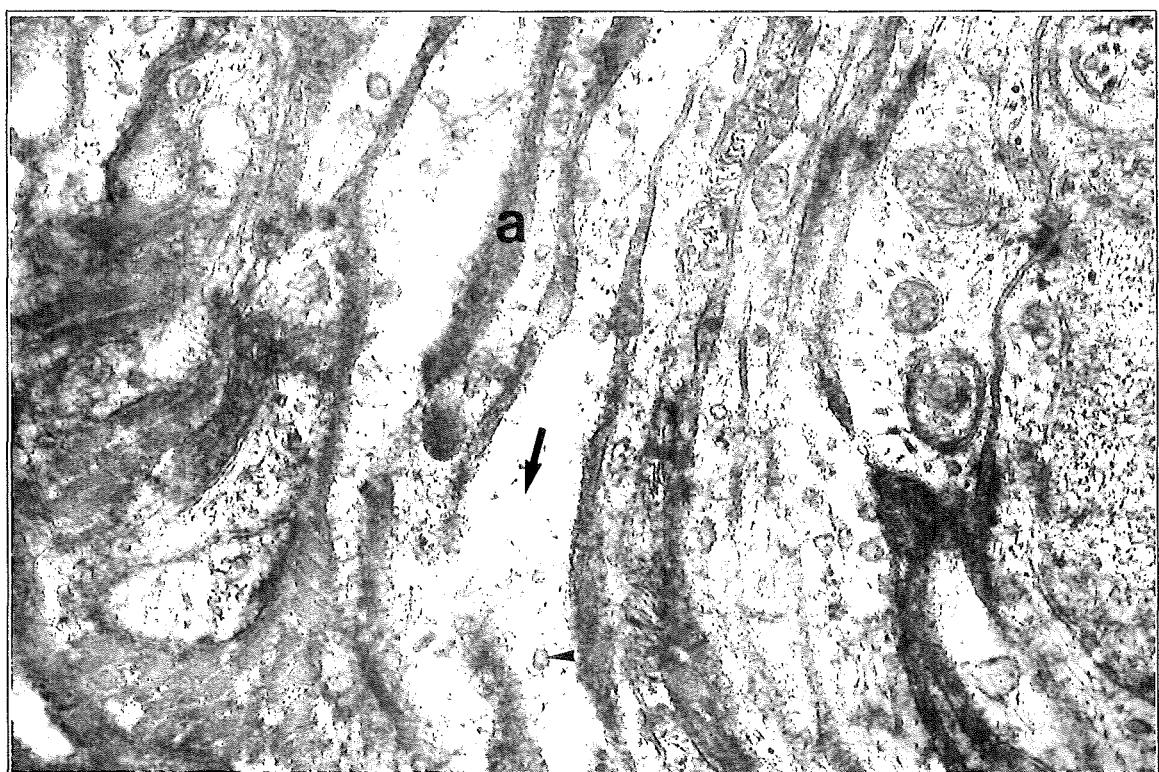


Figure 3.37. 4-week-old calf. A higher magnification of the concentric lamellae enclosing the ultra-terminal axon. The electron-lucent lamellae contain a few strands of filamentous material (arrow) as well as several vesicles (arrowhead). An amorphous electron-dense substance (a) fills the interlamellar spaces. TEM. X 40,000.



Figure 3.38. 4-week-old calf. Transverse section through a bulbous corpuscle located in the reticular dermis close to a nerve fascicle. Two axonal profiles (a) are seen in the middle of the structure. The lower axonal profile appears to have an incomplete myelin sheath (arrowhead). Two inner core lamellae (arrows) enclose the axons. Between the axons and lamellae are longitudinally orientated collagen fibres (c). The capsule is formed by perineural cells (p). TEM. X 8,000.

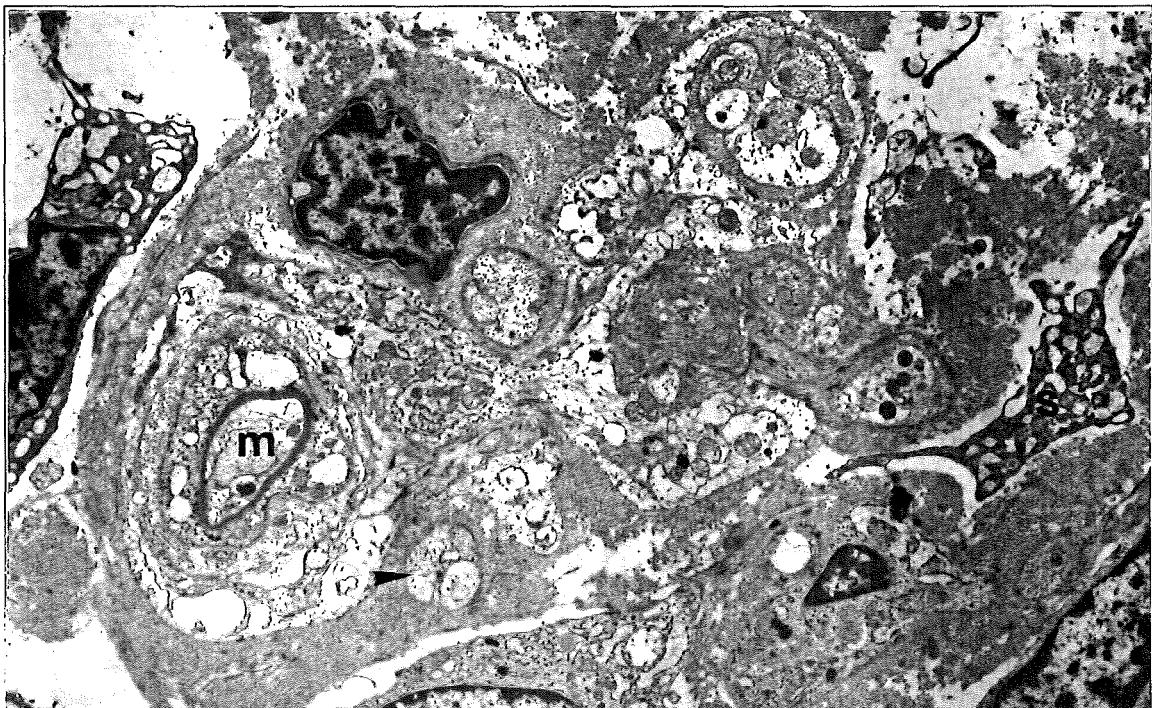


Figure 3.39 2-week-old calf. A myelinated nerve (m) divides within the connective tissue sheath of a hair follicle into several Ruffini terminals, some of which are enclosed in Schwann cell cytoplasm (arrowhead). Septal cell processes (S) divide the surrounding connective tissue into compartments. TEM. X 5,400.

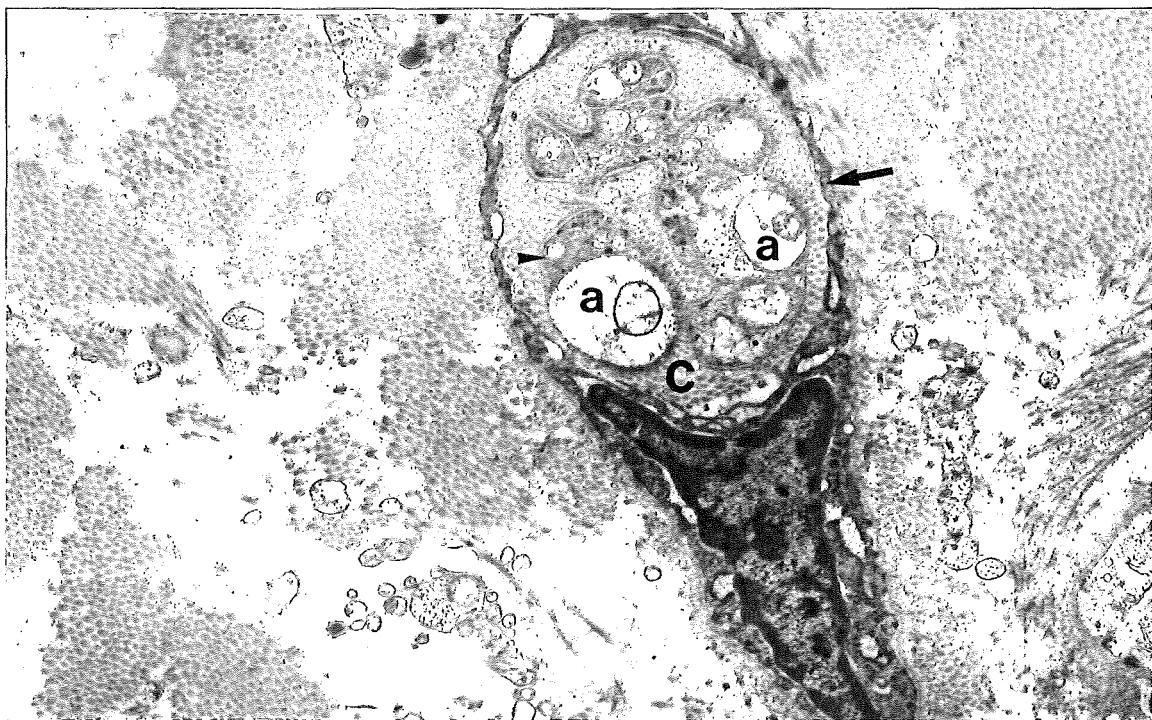


Figure 3.40 2-week-old calf. A higher magnification of a Ruffini corpuscle composed of several axons (a) ensheathed in Schwann cell cytoplasm (arrowhead). Collagen fibres (c) surround the axons. The whole structure is separated from the rest of the connective tissue by a septal cell (arrow). TEM. X 10,000.

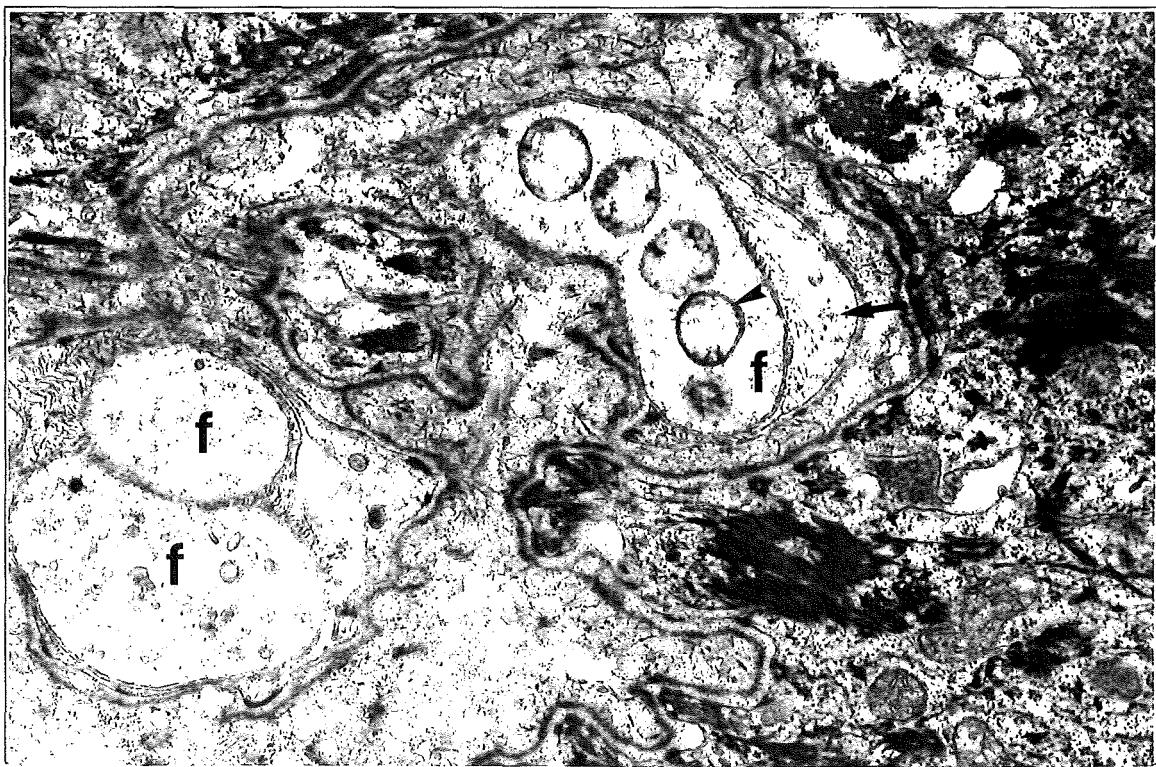


Figure 3.41. 2-day-old calf. A transverse section of the horn epidermis and dermis shows three free nerve endings (f). The largest free nerve ending contains a row of large vesicles (arrowhead), whilst the smaller nerves contain vesicles and filaments. Schwann cell processes enclose the free nerve endings (arrow). TEM. X 20,000.

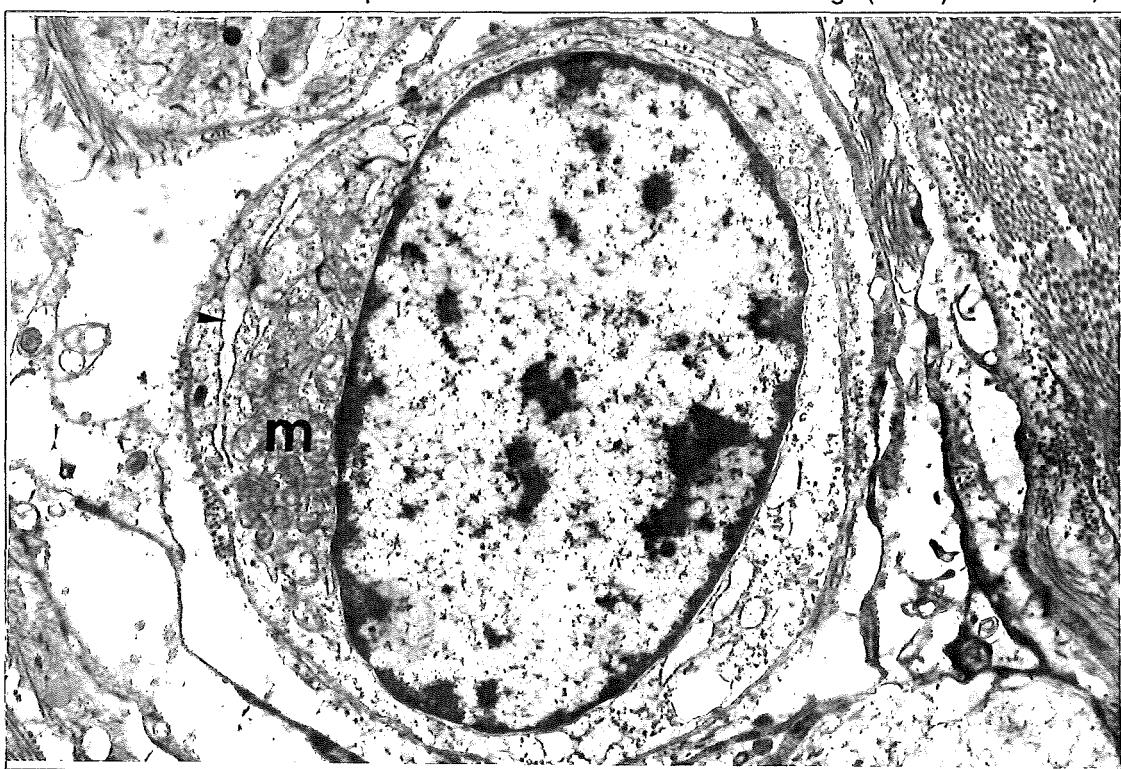


Figure 3.42. 4-week-old calf. A terminal Schwann cell. One side of the cell contains an accumulation of mitochondria (m) and a few rough endoplasmic reticulum profiles (arrowhead). Rough endoplasmic reticulum and electron-dense granules are seen on the opposite side of the cell. TEM. X 10,000.



Figure 3.43. 3-week-old calf. At the base of a dermal papilla, a myelinated nerve fibre sheds its myelin sheath (arrow) before forming an expanded ending (f). Note the terminal Schwann cell (T) near the expanded nerve. TEM. X 4,200.

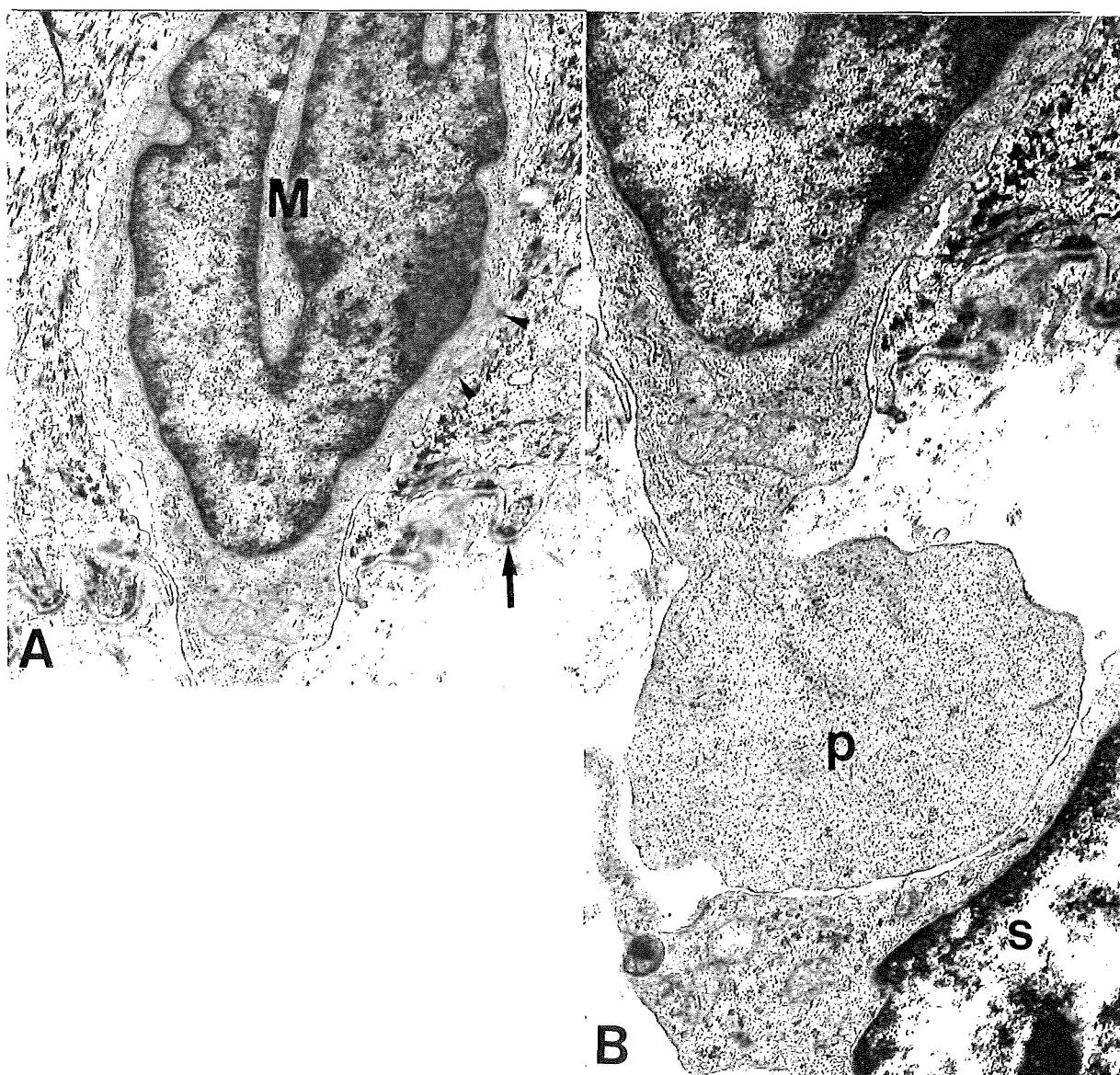


Figure 3.44. 198-day-old bovine foetus. A. A presumptive Merkel cell (M) extends through a gap in the basal lamina (arrow). The Merkel-like cell contains a deeply indented nucleus and is connected to adjacent cells by small desmosomes (arrowheads). TEM. X 13,400.

B. The basal process (P) of the Merkel-like cell makes contact with a Schwann cell (S) which is located in the dermis. Several polyribosomes are scattered throughout the cytoplasm of the Schwann cell. TEM. X 20,000.

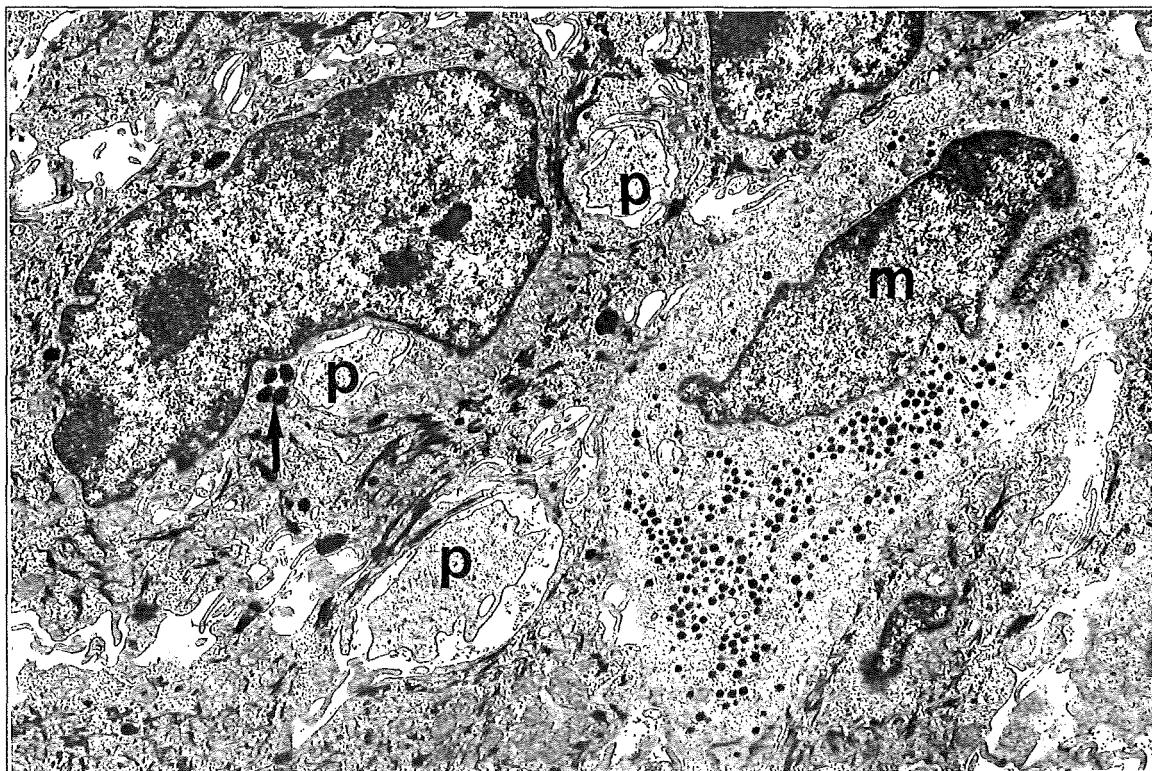


Figure 3.45. 198-day-old bovine foetus. A Merkel cell (m) with small electron-dense bodies and several cytoplasmic processes (p) is seen within the stratum basale. The electron-dense bodies in the Merkel cell are easily distinguished from melanosomes (arrow) in the adjacent keratinocyte. TEM. X 10,000.



Figure 3.46. 2-week-old calf. Transverse section through the stratum basale. An intraepidermal nerve (arrow) is seen invaginating a keratinocyte (k). It is most likely that the intraepidermal nerve originated from the free nerve ending (f) seen in the dermis. Arrowheads mark the basal lamina. TEM. X 12,000.

CHAPTER 4.

**THE DEVELOPMENT OF THE HORN BUD IN
THE PRE-NATAL AND POST-NATAL BOVINE
AND CAPRINE: AN HISTOCHEMICAL AND
ULTRASTRUCTURAL INVESTIGATION.**

INTRODUCTION

It is known that the development of the skin and its derivatives is dependent on inductive interactions between the epidermis and dermis (Cohen, 1969; Maruyama *et al.*, 1988; Du Cros *et al.*, 1992). The disruption of these inductive mechanisms in the horn bud could in theory prevent horn development and thus remove the need for the dehorning procedure. However, in order to effectively disrupt horn growth, it is necessary to understand the normal development of the horn.

Few detailed studies have been carried out on the pre-natal and post-natal development of the ruminant horn. George (1955) and Lyne and Hollis (1973) studied pre-natal and post-natal horn development in Merino sheep. However Lyne and Hollis (1973) concentrated mainly on the development of hair follicles and sebaceous glands, rather than on the morphological changes of cells in the horn region. Apart from the work carried out by George (1955) and Lyne and Hollis (1973) there does not seem to be any recently published material on the morphological changes of the epidermis and dermis in the horn region of ruminants, especially at the electron microscope level. In contrast, there is a great deal of literature concerning the development of other region of skin at the light microscope level in sheep (Hardy and Lyne, 1956; Lyne, 1957), cattle (Lyne and Heideman, 1959), rat (Bonneville, 1968) and cat (Baker, 1974). Most of the studies on the ultrastructural development of skin have been carried out on human foetuses (Hashimoto *et al.*, 1966^b; Breathnach and Robins, 1969; Hashimoto, 1971, 1972; Breathnach, 1971^{a,b}; Holbrook and Odland, 1975), with less research on sheep (Lyne and Hollis, 1971, 1972), pigs (Fowler and Calhoun, 1964; Meyer *et al.*, 1986) and mice (Menefee, 1957; Van Exan and Hardy, 1984; M'Boneko and Merker, 1988). This study was undertaken to provide information on the development of the horn bud in cattle and goats using light, scanning and transmission electron microscopy. The results

obtained from this study will serve as a baseline for future studies on the interactions between the developing horn bud and underlying dermis.

LITERATURE REVIEW

EPIDERMIS

STRATUM BASALE

The stratum basale is composed of a single layer of keratinocytes which rest on a basal lamina (Junqueira *et al.*, 1995). The shape of the cells varies with the region of the body. In areas where thin skin is present the basale cells tend to be cuboidal in shape, whereas columnar basale cells are found in regions with thick skin (Creed, 1958; Goldsberry and Calhoun, 1959). The basal plasma membrane of the keratinocytes is thrown into cytoplasmic processes which interdigitate with the basal lamina (Leeson and Leeson, 1981). Numerous hemidesmosomes are found at the interface of the stratum basale and basal lamina, whilst desmosomes connect the lateral plasma membranes of adjacent keratinocytes. Mitotic figures are frequently seen in this layer, which is responsible for the supply of cells to the more superficial layers. The stratum basale cells contain numerous rod-shaped mitochondria, free ribosomes, polyribosomes and tonofilaments (Cormack, 1987). The high ribosomal content is responsible for the basophilic nature of the cytoplasm. The highest concentration of melanin granules in the epidermis is found in the stratum basale, where the granules typically have a perinuclear location (Creed, 1958).

STRATUM INTERMEDIUM

The stratum intermedium is located between the periderm and stratum basale of the developing foetal epidermis. The stratum intermedium is formed

by large, glycogen-filled cells which subsequently differentiate into the stratum spinosum, stratum granulosum and stratum corneum. The light microscopic structure of this intermediate cell layer was well documented in the first half of the century (Arey, 1941; Hamilton *et al.*, 1946). The ultrastructural morphology of the intermediate cells received less attention until Hashimoto *et al.* (1966^b) described the ultrastructure of skin in 12 to 22-week-old human foetuses. Hashimoto *et al.* (1966^a) later extended their study to include the ultrastructural morphogenesis of intermediate cells in the nail-bed of 16 to 18-week-old human foetuses. Later, Breathnach and Smith (1968) described the development of the intermediate cell layer in the hair germ of human foetuses. The development of the hair germ was compared with the development of interfollicular epidermis. Further studies on the development of the intermediate cell layers have been carried out in the pig (Meyer *et al.*, 1986) and rabbit (Maruyama *et al.*, 1988).

The stratum intermedium is not recognized once distinct spinosum, granulosum and corneum layers have developed.

STRATUM SPINOSUM

The stratum intermedium differentiates into spinosum and granulosum layers between days 18 and 19 of gestation in the mouse (Du Brul, 1972). The stratum spinosum at this stage contains thick tonofilament bundles which are attached to desmosomes (Du Brul, 1972).

Post-natally, the stratum spinosum is composed of several layers of keratinocytes with numerous interdigitating cell processes, which are connected by desmosomes (Williams *et al.*, 1995). In cattle, prominent intercellular spaces are present in regions where more than six spinosal cell layers occur (Goldsberry and Calhoun, 1959). Likewise, in sheep the intercellular spaces are marked in areas of thick epidermis, such as the muzzle and lip (Kozlowski and

Calhoun, 1969). In the dog the number of layers forming the stratum spinosum varies between two and thirty-five depending on the body region (Johnson and Calhoun, 1954).

The spinosum cell cytoplasm contains ribosomes and melanosomes, in addition to tonofilament bundles (Williams *et al.*, 1995). The tonofilaments maintain the structure of the cell layers and resist any abrasive forces on the epidermis (Junqueira *et al.*, 1995).

STRATUM GRANULOSUM

The stratum granulosum is composed of one or more layers of degenerating cells. The number of layers present is dependent on the region of the body. Generally the stratum granulosum is thickest in thick skin, such as the digital pad of the dog (Webb and Calhoun, 1954), as well as the muzzle and hooves of ruminants (Goldsberry and Calhoun, 1959; Kozlowski and Calhoun, 1969). The stratum granulosum tends to be discontinuous in thin skin (Creed, 1958). The granulosum cells contain oval nuclei orientated with their longitudinal axes parallel to the epidermal surface. The cells also contain masses of tonofilaments, orientated parallel to the epidermis, in addition to basophilic keratohyalin granules which are closely associated with the tonofilaments (Brody, 1960; Du Brul, 1972). Keratohyalin granules in the granulosum cells vary greatly in shape and size. The larger granules occur in cells close to the stratum corneum (Brody, 1959). In addition to keratohyalin granules, the stratum granulosum contains oval or rod-shaped membrane-coating granules which discharge their contents into the surrounding intercellular spaces to form an impermeable layer (Junqueira *et al.*, 1995). The membrane-coating granules contain carbohydrate, lipid and hydrolytic enzymes (Williams *et al.*, 1995).

STRATUM LUCIDUM

The stratum lucidum is an acidophilic homogeneous layer found between the strata granulosum and corneum. The cells forming this layer have ill-defined borders and degenerative nuclei (Creed, 1958). The stratum lucidum contains eleidin, which is thought to be a derivative of keratohyalin (Creed, 1958). In cattle a stratum lucidum is present at the hoof margin, as well as in the perianal region (Goldsberry and Calhoun, 1959). In sheep a stratum lucidum is found in the lip, hoof and muzzle (Kozlowski and Calhoun, 1969). The stratum lucidum in the sheep hoof contains horn tubules which are also present in the stratum corneum (Deane *et al.*, 1955).

STRATUM CORNEUM

The thickness of the stratum corneum in cattle is dependent on the region of the body from which the skin sample is taken, as well as on the breed of the animal (Amakiri, 1973). The stratum corneum is composed of several layers of desquamating cells which contain a high proportion of keratin (Creed, 1958). The keratin present in stratum corneum cells is embedded in an amorphous matrix composed of the protein, filaggrin (Williams *et al.*, 1995). During the keratinization process the number of lysosomes in the cytoplasm increases resulting in the degradation of cellular organelles by lysosomal enzymes (Junqueira *et al.*, 1995). Lipid is present between the layers forming the stratum corneum in cattle (Lloyd *et al.*, 1979). The lipid acts as a sealant, to prevent water loss, especially on the surface of the superficial corneal layer where it is deposited along the margins of the cells (Jenkinson and Lloyd, 1979).

PERIDERM

The fetal epidermis is covered by a layer of ectodermally-derived cells known as the periderm. The presence of the periderm has been noted in humans (Breathnach and Wyllie, 1965), rats (Bonneville, 1968), mice (Du Brul, 1972; Nakamura and Yasuda, 1979; M'Boneko and Merker, 1988), sheep (Lyne and Hollis, 1972), and pigs (Meyer *et al.*, 1986). Most of the work on the periderm has been carried out on human foetuses, where the development of the periderm from different regions of the body has been compared (Holbrook and Odland, 1980).

Much debate has surrounded the origin of the periderm. Some authors, such as Bonneville (1968) believed that the periderm had the same origin as the epithelial lining of the amniotic cavity, whilst other researchers (Breathnach and Wyllie, 1965; Hashimoto *et al.*, 1966^b; M'Boneko and Merker, 1988; Byrne *et al.*, 1994) reported that the periderm developed from the stratum basale. The presence of mitotic figures in the periderm indicates that it is a self-sustaining cell layer (Breathnach and Robins, 1969; Holbrook and Odland, 1975). Periderm development is more advanced in regions of the body which have a thick epidermal layer, such as the plantar surface of the foot in humans (Holbrook and Odland, 1980).

In young foetuses the periderm is composed of flattened cells which later become dome-shaped (Hoyes, 1968). The morphology of the periderm does not vary significantly between species. The cells of the periderm are characterized by the presence of numerous surface microvilli, cytoplasmic filaments and pinocytotic vesicles (Breathnach and Wyllie, 1965). In younger foetuses the periderm cells contain numerous cytoplasmic granules, glycogen and well formed Golgi complexes (Breathnach and Robins, 1969). In older foetuses degenerating organelles and numerous filaments are found within the periderm

(Hashimoto *et al.*, 1966^b). In human foetuses the density of microvilli varies with age (Hoyes, 1968) and location (Breathnach and Robins, 1969). A moderately electron-dense filamentous substance covers the microvilli (Hashimoto *et al.*, 1966^b). Hoyes (1967) identified this substance as an acid mucopolysaccharide which is thought to be secreted by the periderm.

In addition to microvilli, protrusions are sometimes seen on human periderm cells. According to Hoyes (1968) the presence of protrusions was first observed by Bowen in 1889. Bowen referred to the protrusions as "bladder cells". The ultrastructural appearance of the protrusions was first studied by Hoyes (1968). Hoyes (1968) suggested that in later stages the protrusions became detached from the underlying periderm cells. The scanning electron microscope appearance of the protrusions was first observed by Whittaker and Adams (1971).

The fate of the periderm has attracted a large amount of interest. Several workers have reported the absence of keratohyalin granules in the periderm indicating that the periderm does not undergo keratinization (Hashimoto *et al.*, 1966^b). More recently cytokeratins have been identified in the periderm of foetal pigs using immunohistochemistry (Meyer *et al.*, 1986). However Meyer *et al.* (1986) have stated that the cytokeratins function in the stabilization of the cell form and do not appear to be involved in keratinization of the periderm. It is accepted that once the underlying cell layer begins to undergo keratinization the periderm undergoes apoptosis, which is a programmed cell death. Apoptosis of the periderm is characterized by the formation of globular structures and a submembranous cell envelope, an accumulation of cytoplasmic filaments and the presence of pyknotic nuclei (Polakowska *et al.*, 1994). In most species the periderm is shed before birth (Du Brul, 1972). However the periderm is retained post-natally in the marsupial, *Trichosurus vulpecula* (Lyne *et al.*, 1970) where it

is thought to protect the neonate from desiccation as it moves from the urogenital opening to the pouch.

The exact function of the periderm is unclear, but it is thought to play a role in the protection of the foetus, as well as in the secretion and absorption of substances from the amniotic fluid. Breathnach and Wyllie (1965) observed the presence of microvilli and cytoplasmic vesicles which they reported indicated that the periderm had a secretory function. They went further to suggest that the substance seen on the surface of the microvilli was a secretion from the periderm cells. They suggested that the substance on the surface of the microvilli was mucus, due to the similarity between periderm cells and mucus secreting metaplastic embryonic chick cells. The substance covering the microvilli was identified as an acid mucopolysaccharide by Hoyes (1967). Later, it was shown that the periderm of foetal mice synthesized and secreted proteins into the amniotic fluid, thus influencing the composition of amniotic fluid (Janzen *et al.*, 1984; M'Boneko and Merker, 1988). Due to the exchange of materials between the periderm and amniotic fluid M'Boneko and Merker (1988) suggested that an inductive interaction existed between the periderm and amniotic fluid, which stimulated the periderm to synthesize and secrete proteins. More recently Byrne *et al.* (1994) have reported that the morphology and functions of the periderm are under the control of inductive influences in the dermis.

An absorptive function for the periderm was suggested by Breathnach and Wyllie (1965). They observed a morphological similarity between the periderm and the syncytial cells of the trophoblast, which are known to have an absorptive role. In addition, Bonneville (1968) also postulated that the periderm was involved in the exchange of substances between the skin and amniotic fluid. The absorptive function of the periderm was supported by Du Brul (1972) who noted that periderm cells in early mice foetuses could take up material from the external environment. It has been reported that the periderm is involved in the

absorption of glucose from the amniotic fluid (Holbrook and Odland, 1975; Verma *et al.*, 1976). The glucose is stored in the periderm as glycogen, which is used to provide energy for the differentiation of the underlying cell layers before the development of the sub-epidermal capillary network (Maruyama *et al.*, 1988).

DERMIS

The dermis is composed of a superficial papillary layer and a deep reticular layer. A sharp demarcation is not present between these two dermal layers in either cattle or dogs (Johnson and Calhoun, 1954; Goldsberry and Calhoun, 1959).

PAPILLARY DERMIS

This layer possesses dermal papillae which interdigitate with epidermal ridges. Dermal papillae are more prominent and complex in non-hairy regions of the body in the dog and cat (Creed, 1958). In cattle and sheep the complexity of dermal papillae is related to skin thickness. In areas, such as the muzzle and hoof margins, where a thick epidermis is present, the dermal papillae are well-formed and branched (Goldsberry and Calhoun, 1959; Kozlowski and Calhoun, 1969). Collagen, elastic and reticular fibres form the connective tissue framework of the papillary dermis. In sheep elastic fibres are more numerous in the papillary dermis than in the reticular dermis (Kozlowski and Calhoun, 1969). In addition to connective tissue fibres, the papillary dermis in the dog and cat contains fibroblasts, mast cells, melanocytes and macrophages (Creed, 1958). Large numbers of mast cells, some in groups, are present in the papillary dermis of sheep (Kozlowski and Calhoun, 1969). In cattle the papillary layer contains clumps of lymphocytes, plasma cells and eosinophils (Goldsberry and Calhoun, 1959).

RETICULAR DERMIS

The reticular layer contains thick collagen bundles with a few cells interspersed amongst the bundles. In the dog elastic fibres are more numerous in the reticular layer than in the papillary layer (Johnson and Calhoun, 1954; Creed, 1958). However, in cattle few elastic fibres are present in the reticular layer (Goldsberry and Calhoun, 1959). In cattle the reticular dermis is composed of thick collagen bundles which are orientated parallel to the skin surface (Goldsberry and Calhoun, 1959).

MATERIALS AND METHODS

The bovine foetuses used in this study were obtained from Glasgow and Paisley abattoirs. The foetuses were predominantly of Friesian and Ayrshire breeds. Crown-rump lengths, sexes and estimated days of gestation of the foetuses used in this study are shown in Table 4.1. Eight calves ranging in age from 2 days to 4 weeks were also used in this study. In addition, ten kids aged between 10 hours and 8 weeks of age were obtained from a farm in Aberfoyle. Details of the calves and kids are shown in Tables 2.3 and 2.4 of Chapter 2. The developing horn tissues were processed for light microscopy, scanning electron microscopy and transmission electron microscopy.

Table 4.1. Details of bovine foetuses used for light microscopy, scanning and transmission electron microscopy.

Estimated Gestational Age (days)	Crown-Rump Length (mm)	Sex
71	96	male
104	210	female
112	245	male
117	260	female
122	280	female
125	290	female
146	350	female
154	395	male
159	430	female
169	480	male
182	530	female
198	580	female
203	595	female
204	605	female
206	625	male

LIGHT MICROSCOPY

The developing horn bud tissue was fixed in neutral buffered formalin for 5 days. This was followed by post-fixation in mercuric chloride for 3 days. The mercuric chloride solution was made up as follows:

Saturated aqueous mercuric chloride 900ml.

Formalin 100ml.

After impregnation with paraffin wax, the tissues were sectioned at 5 μ m. The sections were then stained with H&E and Masson's trichrome.

The Periodic-Acid-Schiff (PAS) technique was used to demonstrate the presence of glycogen in pre-natal bovine horn. Control slides, treated with diastase, were used to ensure that the PAS was specific for glycogen. Oxidation was carried out in 1% aqueous periodic acid for 5 minutes, after which the

sections were rinsed in water for a further 5 minutes. The sections were then flooded with Schiff's reagent. After 20 minutes the reagent was washed off. The sections were then counter-stained with celestine blue, dehydrated, cleared and mounted.

The presence of glycogen was indicated by red-staining material in sections not treated with diastase.

SCANNING ELECTRON MICROSCOPY

The processing procedure for scanning electron microscopy is detailed in the Chapter 1, General Materials and Methods. After processing, horn bud sections were mounted on stubs with the cut surfaces uppermost (Figure 4.1). After viewing this surface the pre-natal tissues were reorientated on the stubs so that the periderm was uppermost (Figure 4.1). The tissues were then recoated with gold-palladium and viewed with the scanning electron microscope.

TRANSMISSION ELECTRON MICROSCOPY

The tissues were minced in Karnovsky's fixative. The tissues were then post-fixed in 1% osmium tetroxide, dehydrated through a series of graded alcohols, cleared in propylene oxide and embedded in Araldite as detailed Chapter 1.

LIGHT MICROSCOPY RESULTS

PRE-NATAL DEVELOPMENT IN CATTLE

71 DAYS OF GESTATION

Epidermis

In the horn bud region a single layer of tall columnar cells formed the stratum basale. The columnar basale cells contained oval-shaped nuclei which were surrounded by a thin rim of cytoplasm. In areas of hair germ proliferation two to three layers of cuboidal cells were present. Large amounts of glycogen were demonstrated in these areas of hair germ proliferation, whilst only traces of glycogen were present in the tall columnar basale cells.

The stratum intermedium overlying the stratum basale was composed of three layers of large, round cells with apically-placed nuclei and clear cytoplasm. The two superficial intermedium cell layers contained a moderate amount of basally-located glycogen, whilst the smaller cells in the deep intermedium layer had a few localized areas of glycogen (Figure 4.2A).

Superficial to the stratum intermedium in the horn bud region was a periderm layer composed of cells with voluminous central regions and flattened peripheral regions. Very few binucleated periderm cells were seen at this stage of horn bud development. A large amount of glycogen was distributed throughout the periderm cytoplasm.

At this stage the epidermis of the adjacent skin was the same thickness as in the horn area. However, the cells forming the skin contained little glycogen in comparison to the horn bud cells (Figure 4.2B; Table 4.2).

Dermis

The dermis underlying the horn bud contained a large number of cells interspersed with a few fibres. The cells in this region were a combination of fibroblasts and mesenchymal cells. The stellate-shaped mesenchymal cells had oval-shaped nuclei surrounded by a small amount of cytoplasm which extended into several thin processes. The fibroblasts contained elongated nuclei enclosed in abundant basophilic cytoplasm. Several layers of spindle-shaped fibroblasts with dark elongated nuclei were seen in the sub-epidermal region. The fibroblasts were orientated with their longitudinal axes parallel to the horn bud surface. The highest concentration of cells was seen below the centre of the horn bud. In addition to the mesenchymal cells and fibroblasts, several cells with dark, round nuclei, surrounded basophilic cytoplasm, were found in the papillary dermis (Figure 4.3). It was not possible to identify these cells solely on the basis of their light microscopic appearance, but the Transmission Electron Microscopy study suggested that these cells were mast cells. The papillary dermis contained a large number of dilated blood vessels.

The adjacent skin contained mainly mesenchymal cells, with large, oval-shaped nuclei and scant cytoplasm. The papillary dermis in the skin was less cellular than the papillary dermis underlying the horn bud. Blood vessels were present in the papillary dermis of the skin, but were not situated close to the epidermis as had been observed in the horn bud.

104 DAYS OF GESTATION

Epidermis

The stratum basale consisted of two to four layers of tall columnar cells, which contained oblong nuclei and scant basophilic cytoplasm. Several melanocytes with dark granules and long cytoplasmic processes were observed

in the stratum basale, as well as in the sub-epidermal region. Cuboidal cells formed the stratum basale in the adjacent skin. Hair germ proliferation in the horn region was advanced, with all the hairs angled towards the centre of the horn. At this stage hair germ initiation had begun in the stratum basale of the adjacent skin.

The stratum intermedium in the horn region contained twelve to fourteen cell layers of large, round cells. The size of the intermedium cells decreased basally. Amongst the intermedium cells were small cells with dense, central nuclei enclosed in eosinophilic cytoplasm (Figure 4.4A). These cells were thought to be Langerhans cells due to their location within the stratum intermedium. Furthermore Langerhans cells were identified ultrastructurally in the stratum intermedium of this foetus. The stratum intermedium of the adjacent skin was three to four cell layers thick (Figure 4.4B).

Flat cells containing eosinophilic cytoplasm formed the periderm in the horn region. Many of the periderm cells were binucleated.

Dermis

The papillary dermis of the horn bud contained a mixture of fibroblasts and mesenchymal cells with the former dominating. Whereas the horn bud region was dominated by fibroblasts with large, elongated nuclei, the adjacent skin contained cells with small, round dark nuclei and scant basophilic cytoplasm which were identified in the Transmission Electron Microscopy section as undifferentiated mesenchymal cells.

A few poorly developed collagen fibres were demonstrated at this stage of development in the papillary dermis of the horn bud. Well-developed loose, irregular collagen bundles were observed in the reticular dermis, whilst more regularly orientated collagen bundles were present in the deep dermis. The

reticular dermis contained a dense collection of blood vessels which discharged branches into the papillary dermis. A few mitotic figures were observed in fibroblasts of the deep dermis. A muscle layer was observed in the deep dermis underlying the horn bud and skin.

112-117 DAYS OF GESTATION

Epidermis

Tall columnar cells formed the stratum basale, except in areas of hair germ proliferation where clusters of cuboidal cells with round, pale-staining nuclei were found. Mitotic figures were frequently observed in the hair germs. At this stage melanocytes with long, dendritic processes were seen in contact with the basal regions of the stratum basale cells (Figure 4.5). Furthermore several cells in the basale and intermediate layers contained melanin granules.

Eight cell layers formed the stratum intermedium in the horn bud region. The intermediate cell layer directly below the periderm consisted of large, round cells which contained apically positioned nuclei enclosed in a network of tonofilaments. The size of the intermedium cells decreased basally, with the smallest cells being in contact with the stratum basale. The stratum intermedium in the horn bud contained less glycogen than in the 71 days old foetus. The glycogen in the stratum intermedium was distributed in a reticular pattern throughout the cell (Figure 4.6).

Two layers of periderm cells were observed in some regions of the horn bud. The periderm in the horn bud was formed by a combination of spindle-shaped and irregular-shaped cells (Figure 4.6). The periderm nuclei were centrally located and varied in shape from elongated to round. Most of the periderm cells possessed large amounts of evenly distributed glycogen.

Dermis

Few hair follicles were seen in the horn bud centre. Long, well-developed hair follicles, which were angled towards the centre of the horn bud, were seen in the outer limits of the horn bud. Loose collagen fibres interspersed with fibroblasts and mesenchymal cells were observed in the papillary dermis. This contrasted with the thick, irregular collagen bundles observed throughout the reticular and deep regions of the dermis in the horn bud. During this period of development the fibroblasts in the reticular and deep dermal layers had become orientated with their longitudinal axes parallel to the collagen bundles. Occasional dividing cells were seen in the reticular and deep regions of the horn bud dermis (Figure 4.7). These cells were assumed to be fibroblasts due to their position close to the collagen bundles and the presence of abundant basophilic cytoplasm.

Muscle fibres were scattered in the deep dermis underlying the horn bud at 112 days of gestation, but by 117 days muscle was restricted to the skin surrounding the horn bud.

122-125 DAYS OF GESTATION

Epidermis

A combination of tall columnar and low cuboidal cells formed the stratum basale in the horn bud region. The stratum basale cells contained relatively little glycogen. At this stage the stratum intermedium in the central region of the horn bud was composed of five cell layers, as was the stratum intermedium of the adjacent skin. However, the stratum intermedium at the edges of the horn bud consisted of twenty to twenty-six cell layers. The hair follicles in the horn bud region were well developed. Few new hair germs were present in the horn bud region. Hair canal formation had begun, with clusters of cells observed in the

lower layers of the stratum intermedium in the horn bud region. In the stratum intermedium of the horn bud, glycogen was restricted to the peripheral regions of the cells.

The periderm, covering the central regions of the horn bud was composed of a discontinuous layer of rectangular cells with moderately eosinophilic cytoplasm. The edges of the horn bud had large irregular-shaped periderm cells. Most of the periderm cells, covering the edges of the horn bud, had two to three nuclei. Glycogen was still present in the periderm covering the horn bud.

A flattened layer of periderm cells covered the skin of the adjacent frontal region. A small amount of glycogen was found in the periderm of the adjacent skin, but was absent from the other cell layers.

Dermis

Developing hair follicles were distributed throughout the horn bud region. Sebaceous glands were at an early stage of development with out-pocketings visible on only a few hair follicles. Thin, randomly arranged collagen fibres were observed in the papillary dermis of the horn bud. A few mesenchymal cells, fibroblasts and lymphocytes were found in this area. In contrast, the papillary dermis of the surrounding skin was highly cellular, with mesenchymal cells and fibroblasts interspersed between the developing hair follicles. The horn bud reticular dermis contained loose, irregular collagen bundles, whilst that of the adjacent skin was composed of dense, irregular collagen. The deep dermis in both the horn bud and skin was a relatively thin layer made up of dense collagen bundles. Spindle-shaped fibrocytes were the predominant cell type in the deep dermis.

146 DAYS OF GESTATION

Epidermis

Columnar cells with long basal cytoplasmic extensions formed the stratum basale in the horn bud region. The stratum intermedium in this region contained nine layers, whilst the stratum intermedium of the adjacent skin was composed of four to five cell layers. The horn bud surface in the 146 day foetus had an uneven scalloped appearance due to the presence of a discontinuous layer of irregular-shaped periderm cells (Figure 4.8). In contrast, flat, uniform-sized periderm cells covered the skin surrounding the horn bud. Binucleated periderm cells containing eosinophilic cytoplasmic granules were present in the horn bud region.

Dermis

The papillary dermis directly below the horn bud epidermis still contained thin collagen fibres. However, well-developed, densely-packed collagen bundles surrounded the developing hair bulbs in the lower regions of the papillary dermis. Most of the hair follicles in the horn bud area had developing sebaceous glands with round, vacuolated cells.

Fibroblasts, maturing mesenchymal cells and undifferentiated mesenchymal cells with small, dark nuclei were scattered throughout the papillary dermis of the horn bud. Few blood vessels were seen close to the epidermis in the horn bud region, although several blood vessels were present at the level of the hair bulbs. In the neighbouring skin, numerous blood vessels were observed in the papillary layer close to the epidermis.

The reticular dermis in the horn region was composed of irregularly-arranged loose collagen bundles, which surrounded large blood vessels and nerves. A combination of fibroblasts and fibrocytes lined the collagen bundles.

The fibroblasts contained pale-staining elongated nuclei, whilst the fibrocytes were identified by the presence of elongated dark nuclei. The collagen bundles in the deeper dermal regions were arranged in bands orientated parallel to the epidermal surface. The surrounding skin had a thick cutaneous muscle layer which did not extend below the horn bud.

154 DAYS OF GESTATION

Epidermis

A combination of cuboidal and columnar cells formed the stratum basale in the horn bud. At this stage the stratum intermedium in the horn bud contained nine cell layers, whilst that of the skin had six layers. Hair canals extended as far as the top layer of the stratum intermedium in the horn bud. The hair canals were lined by two to three layers of keratinized cells (Figure 4.9). The superficial stratum intermedium layer consisted of large cells containing numerous eosinophilic granules. Overlying this granular layer were flat, desquamating periderm cells.

The whole horn bud was Periodic Acid Schiff-positive, with the most intense staining located in the periderm cells. Dense masses of glycogen were seen in some of the deeper stratum intermedium cells (Figure 4.10).

Dermis

The papillary dermis of the horn bud contained fibroblasts and a few undifferentiated mesenchymal cells with small, dark nuclei. Several capillaries were observed close to the epidermis. The papillary dermis of the skin contained dark, spindle-shaped fibroblasts. Few capillaries were seen in the papillary dermis of the skin.

159 DAYS OF GESTATIONEpidermis

The stratum basale contained tall columnar cells with large, dark nuclei surrounded by a thin rim of granular cytoplasm. Large hair germ cells, with pale-staining nuclei and abundant pale cytoplasm were scattered throughout the basale layer.

At this stage the stratum intermedium was divisible into spinosum, granulosum and corneum layers. The superficial layer of the epidermis consisted of desquamating periderm cells. Below this periderm layer was a thin keratinized stratum corneum. Underlying the stratum corneum was a discontinuous stratum granulosum which consisted of flattened cells with granular cytoplasm.

The stratum spinosum below the stratum granulosum consisted of ten cell layers, the uppermost of which contained large, round cells with clear cytoplasm and apically placed nuclei. The remainder of the stratum spinosum contained smaller, more adult-like cells with basophilic cytoplasm.

169 DAYS OF GESTATIONEpidermis

The stratum basale in the horn bud contained tall columnar cells with round nuclei and granular, basophilic cytoplasm. Large hair germ cells with abundant basophilic cytoplasm and large central nuclei were present in the stratum basale. The stratum basale contained glycogen in regions of hair germ development.

Five layers of stratum spinosum cells were found above the basale cells. The two superficial spinosal layers still contained large, immature cells, whereas

the deeper layers contained small adult-like cells (Figure 4.11). A discontinuous stratum granulosum, with centrally located round nuclei and granular cytoplasm was found above the stratum spinosum.

The stratum corneum was continuous with the keratinized lining of the hair canals. In some areas keratinized cells were intermingled with the peridermal cells. The periderm contained a relatively small amount of glycogen.

Dermis

The papillary dermis of the horn bud and skin contained similar cell types. The cells were mainly fibroblasts. The papillary dermis of the skin contained a greater density of fibres compared to the papillary dermis of the horn bud region.

182-206 DAYS OF GESTATION

Epidermis

Cuboidal cells with dark nuclei formed the stratum basale in both the horn bud and skin. During this period of development the stratum spinosum was two cell layers thick, as was the spinosal layer of the adjacent skin (Figure 4.12). The spinosum cells had large round pale-staining nuclei and eosinophilic cytoplasm. The cell boundaries of the stratum spinosum cells were indistinct. On top of the stratum spinosum was a single granulosum layer formed by eosinophilic granular cells. A desquamating periderm layer was present above the stratum corneum in some areas.

During this period glycogen was not observed in the horn bud epidermis, except along the basal lamina and within the hair follicle. However, moderate amounts of glycogen were demonstrated in the stratum spinosum and stratum basale of the surrounding skin.

A summary of the results is shown in Tables 4.2 and 4.3.

Dermis

No difference in the dermal layers of the horn bud and skin were observed. Loose irregular connective tissue was present in the papillary dermis of both areas. The cells in the papillary layer were a mixture of fibroblasts and fibrocytes. The reticular dermis contained thick collagen bundles which were orientated parallel to the hair follicles. The deep dermis comprised of regularly arranged collagen bundles.

Table 4.2. Presence of glycogen in the epidermis of the horn bud and skin in bovine foetuses.

Estimated gestational age (days)	Periderm layer		Stratum intermedium		Stratum basale	
	Horn	Skin	Horn	Skin	Horn	Skin
71	+++	+	++	-	+	-
112-117	+++	+	+	-	+ *	-
122-125	+++	+	+	-	+	-
154	++	+	++	-	+	+ *
159	++	+	+	-	+	+
169	+	-	+++	-	+ *	+ *
182-206	-	-	-	++	+ *	+ *

+++ = Intense staining for glycogen.

++ = Moderate staining for glycogen.

+ = Faint staining for glycogen.

+* = Glycogen present in areas of hair germ initiation.

- = No glycogen demonstrated.

Table 4.3. Number of cell layers present in the horn bud and skin of bovine foetuses

Estimated gestational age (days)	Periderm layer		Stratum intermedium		Stratum basale	
	Horn	Skin	Horn	Skin	Horn	Skin
71	1	1	3	3	1	1
104	1	1	12-14	3-4	2-4	1
112-117	1-2	1	8	4	1	1
122-125	1	1	5-26	5	1	1
146	1	1	9	4-5	1	1
154	1	1	9	6	1	1
159	*	1	11	5	1	1
169	*	1	6	3	1	1
182-206	0	1	3	3	1	1

* = Discontinuous periderm layer

POST-NATAL DEVELOPMENT IN CATTLE

2 DAYS OLD

The horn bud was a raised, dome-shaped area. The horn bud epidermis was only slightly thicker than that covering the adjacent skin. However, the stratum corneum of the horn bud was the same thickness as that of the surrounding skin. The deeper regions of epidermis in the horn bud region formed complex epidermal ridges, whilst the epidermis of the surrounding skin had simple, unbranched epidermal ridges. Five to eight layers of granular cells were present in the horn bud region compared to only two to three in the skin of the frontal region.

No distinction could be made between the dermal regions of the horn and skin.

2-3 WEEKS OLD

A thick stratum corneum was present in the horn bud region. The epidermis underlying the stratum corneum had increased to approximately forty cell layers, compared to the nine layers seen in the adjacent skin. The number of granular cell layers in the horn bud had increased to twenty-three. In the horn bud, long dermal papillae interdigitated with epidermal ridges. Horn tubules were forming from cells located over the tips of the dermal papillae. The epidermal cells forming the horn tubules contained unstained cytoplasm and crescent-shaped nuclei (Figure 4.13). Several degenerating hair follicles and sebaceous glands were present in the dermis below the horn bud epidermis. Many arterio-venous anastomoses were present in the horn bud papillary dermis, but few were observed in the adjacent skin. Isolated sweat glands, characterized by oblong-shaped tubules lined by low columnar cells, were observed in the reticular dermis of the horn bud. Numerous sweat glands were seen in adjacent skin. The reticular dermis of the horn bud contained loose, irregular connective tissue formed by thin collagen bundles, whereas the dermis of the adjacent skin contained thick collagen bundles with little matrix between the bundles.

4 WEEKS OLD

A thick stratum corneum was present in the horn bud. A distinct stratum lucidum separated the thick stratum corneum from the underlying stratum granulosum. Long dermal papillae divided the epidermis into wide epidermal ridges. The columns of cells above the dermal papillae lacked the closely-packed keratohyalin granules seen in adjacent cells. The reticular dermis contained remnants of hair follicles and glands.

POST-NATAL DEVELOPMENT IN GOATS**10 HOURS OLD**

The epidermis increased in thickness towards the centre of the horn. Underlying the stratum corneum was a thick stratum lucidum composed of degenerating cells with pyknotic nuclei and indistinct cell boundaries.

Long dermal papillae extended into the granular layer of the epidermis. From the tips of the dermal papillae arose horn tubules which were formed by cells with dark nuclei and slightly granular cytoplasm (Figure 4.14). The stratum granulosum between the dermal papillae contained a dense concentration of keratohyalin granules. The stratum basale was interspersed with several melanocytes which sent long cytoplasmic processes between the cells of the stratum spinosum.

The horn bud dermis contained a few hair follicles with sebaceous glands. Most of the sebaceous glands were located at the outer limits of the horn bud. The papillary dermis, in the horn bud region, was highly cellular with a mixture of fibroblasts and fibrocytes. The reticular dermis contained a greater proportion of spindle-shaped fibrocytes. Located in the deep regions of the dermis were granular cells which resembled melanocytes. These cells were much larger than typical fibroblasts and possessed long cytoplasmic processes which were filled with dark granules.

2 WEEKS OLD

A thick stratum corneum covered the central region of the horn. The stratum lucidum, underlying the stratum corneum, contained horn tubules. Cross-sections at the level of the stratum granulosum showed the spiral arrangement of the vesicular cells forming the horn tubules. The stratum spinosum in the horn region consisted of oval-shaped cells with prominent

intercellular bridges. Melanin-laden dendrites extended between the stratum spinosum cells. Several melanocytes, surrounded by clear spaces, were seen within the basal cell layer. The melanocytes were more prominent than in the 10-hour-old kid.

The dermis was thickest in the middle of the horn. The papillary dermis contained vertically orientated collagen bundles lined by fibrocytes with dark nuclei and basophilic cytoplasmic processes. Degenerating hair follicles and sebaceous glands were found in this region. Several dermal melanocytes, containing large brown granules, were observed in the papillary dermis.

The reticular dermis contained loose irregular connective tissue amongst which were large blood vessels and thick nerve bundles. The cutaneous muscle layer in the surrounding skin did not extend below the horn.

3 WEEKS OLD

The keratinized horn layer had increased in thickness and was organized into distinct tubular and intertubular zones. The tubular zones were formed by the pale-staining cells seen in younger kids. In the superficial regions of the horn the horn tubules were composed of rows of amorphous eosinophilic material. Close to the tips of the dermal papillae the horn tubules were formed by cells at various stages of degeneration. Initially large vacuoles accumulated in the cytoplasm. This was followed by nuclear disintegration and later by the presence of dark granules in an amorphous eosinophilic mass.

4-6 WEEKS OLD

During this stage of horn development the horn tubules were seen as empty tubular structures with diagonal shelves. The intertubular regions within the stratum corneum consisted of closely packed cells with nuclear debris.

SCANNING ELECTRON MICROSCOPY RESULTS

PRE-NATAL DEVELOPMENT IN CATTLE

71 DAYS OF GESTATION

In vertical sections the cells of the stratum intermedium, in both the horn bud and the skin, contained apical, irregular-shaped nuclei. Tonofilaments were not observed in the intermedium cells at this stage of development. The periderm cells covering the horn bud had central, voluminous regions flanked by lateral attenuated regions. The surrounding skin was covered by elongated periderm cells with a uniform depth throughout the cell.

When the horn surface was viewed it was found that the periderm cells were covered by layers of irregular flakes (Figure 4.15). The flakes did not extend beyond the horn bud which was separated from the adjacent skin by a circular groove. At this stage polygonal-shaped periderm cells covered the adjacent skin. The skin periderm cells had raised borders which were lined with microvilli.

104 DAYS OF GESTATION

At this stage the horn bud was an elongated ridge partially surrounded by a deep groove. The horn bud surface was composed of polygonal-shaped periderm cells. The base of the horn bud possessed a few protrusions which were attached to the margins of the periderm cells. The deep groove at the base of the horn bud contained a dense collection of protrusions which obscured the periderm cells (Figure 4.16). In areas where these protrusions were less dense, they were seen to arise from both the central and marginal regions of the periderm cells. The protrusions were sparsely covered with microvilli which varied in density and arrangement.

112 to 117 DAYS OF GESTATION

At 112 days of gestation the horn bud was a raised uneven area separated from the skin by a circular groove. A mixture of flat and dome-shaped cells formed the periderm covering the horn bud. The periderm covering the sides of the horn bud had single or grouped oval protrusions which obscured the cellular margins (Figure 4.17). The ends of the protrusions were seen to coalesce in some areas. The greatest concentration of protrusions was seen in the 115-day-old foetus. In this foetus the protrusions totally obscured the periderm cell margins. At a higher magnification, the protrusions were seen to possess microvilli of varying densities.

125 DAYS OF GESTATION

The horn bud was a raised uneven mound dotted with cavities in areas where periderm cells had been shed (Figure 4.18). Even at this stage of development the shallow groove surrounding the horn bud had protrusions attached to the periderm margins. There were however, fewer protrusions than in the previous age groups. In addition to the protrusions several cavities were found within the circular groove.

In vertical sections of the edges of the horn bud, the periderm consisted of a single layer of irregular-shaped cells with tapered basal regions (Figure 4.19). Periderm cells in the skin adjacent to the horn bud consisted of a single layer of flat cells (Figure 4.20). The periderm cells in the horn region contained dense cytoplasm with only a few tonofilaments present (Figure 4.19). In contrast the voluminous central regions of skin periderm cells contained numerous tonofilament bundles (Figure 4.20).

The stratum intermedium cells at the edges of the horn bud were more closely packed compared to the cells at the centre of the horn bud. The intermediate cells at the horn bud edges contained tonofilaments throughout the cytoplasm (Figure 4.19). This contrasted with the intermedium cells from the centre of the horn bud which did not have tonofilaments in the perinuclear regions (Figure 4.21). Similar cells were identified in the Transmission Electron Microscopy section.

154-169 DAYS OF GESTATION

The periderm cells and stratum corneum cells covering the horn bud centre were surrounded by spirally arranged hairs (Figure 4.22).

When the vertical cut surface of the horn bud was viewed it was seen that the upper layers of the stratum intermedium contained round to oval cells with apical nuclei and thick tonofilaments. The lower layers of the stratum spinosum had an adult morphology. These small cuboidal cells, with central nuclei were arranged in columns.

TRANSMISSION ELECTRON MICROSCOPY RESULTS

PRE-NATAL DEVELOPMENT IN CATTLE

71 DAYS OF GESTATION

Stratum basale

Wide intercellular spaces separated the basale cells in the horn bud region (Figure 4.23). Long cytoplasmic processes from the lateral regions of the cells extended into the intercellular spaces where they made contact with similar

structures from adjacent cells. A few desmosomes connected these slender cytoplasmic processes. Desmosomes linked the apical borders of the basale cell with the intermediate cell layer. The basale cells contained numerous ribosomes which occurred either singly or in groups. Mitochondria were observed throughout the cells, but the majority were concentrated in the basal regions of the cells. A well-developed Golgi complex, surrounded by numerous vesicles was observed below the nucleus. Small, clear vesicles were seen throughout the cell, especially near the plasma membrane, where evidence of pinocytosis was observed. Several dilated rough endoplasmic reticular profiles were scattered throughout the cytoplasm. Occasional basale cells contained glycogen granules which were concentrated in the basal regions of the cells. In addition, intercellular glycogen was observed between a few of the basale cells (Figure 4.23). Numerous hemi-desmosomes connected the gently undulating basal plasma membrane to the underlying basal lamina.

Stratum intermedium

The stratum intermedium cell layers were composed of large round cells. These cells contained apically located irregular-shaped nuclei which often appeared as two separate segments. A moderate amount of electron-dense cytoplasm enclosed each nucleus. A broad pedicle attached the perinuclear cytoplasm to a peripherally placed rim of electron-dense cytoplasm which contained numerous ribosomal aggregations. Several mitochondria, tonofilaments and membrane-bound vesicles were observed in the peripheral cytoplasm.

The less electron-dense cytoplasm in the central regions of the cell contained clumps of glycogen, most of which were concentrated in the basal areas of the cell (Figure 4.24). In the apical regions of the intermedium cells the glycogen was arranged in anastomosing cords. A few tonofilaments with dense

regions were scattered in the middle part of the cell. Occasionally electron-dense bodies, which resembled keratohyalin granules, were observed (Figure 4.24).

Periderm

The periderm surface was modified by several short microvilli. A "fuzzy" electron-dense coat covered the periderm surface. The superficial areas of the periderm cells were linked by short desmosomes. Two types of periderm cells were recognized in the horn bud region. The more numerous electron-dense periderm cells contained large amounts of glycogen, whilst the occasionally seen electron-lucent cells contained tonofilaments bundles.

The glycogen-laden periderm cells had deep central regions surrounded by flattened lateral regions (Figure 4.25 A). The peripheral regions of these cells were occupied by dense tonofilament bundles. Glycogen granules were scattered in the middle of this peripheral layer, where the tonofilaments were less dense (Figure 4.25 B). The voluminous central region of the periderm cells was dominated by glycogen granules linked together to form a loose lattice. Wide spaces separated the cords of glycogen.

The glycogen-laden periderm cells contained elongated, eccentrically placed nuclei which had several regularly-placed deep clefts (Figure 4.26 A). An occasional vesicle was seen in the clefts. The nuclei were enclosed in electron-dense cytoplasm which contained numerous free ribosomes as well as a few vesicles of various sizes. The membrane-bound vesicles contained moderately electron-dense material. Larger membrane-bound vesicles were seen along the lateral and basal borders of the periderm cells. These vesicles contained eccentrically-placed electron-dense bodies surrounded by granular material (Figure 4.26 B).

Long villous processes were found in areas where the glycogen-laden periderm cells contacted electron-lucent periderm cells and intermediate cells (Figure 4.26 A&B). Short desmosomes connected neighbouring periderm cells to each other, as well as to underlying intermediate cells. The desmosomes were well-developed and consisted of electron-dense plaques on opposing membranes separated by intercellular spaces which contained moderately-dense material. Thick tonofilament bundles attached to the desmosome plaques. No periderm cells containing large amounts of glycogen were observed in the adjacent skin.

The electron-lucent periderm cells in the horn bud region were irregular in shape with thick processes, which interlocked with adjacent cells. The electron-lucent periderm cells in the horn bud tended to be larger than the glycogen-laden periderm cells. Underlying the surface membrane of the electron-lucent periderm cell was a mesh of tonofilaments, amongst which were numerous irregular-shaped pinocytotic vesicles (Figure 4.27 A&B).

Loose tonofilaments and a few aggregations of glycogen were scattered throughout the middle region of the electron-lucent periderm cell. An irregularly-shaped nucleus enclosed in electron-dense cytoplasm was located in the basal region of the cell. The electron-dense cytoplasm contained free ribosomes, clear vesicles, tonofilaments and a few glycogen granules. The nucleus was connected to the basal plasma membrane by a loose network of tonofilament bundles. Several vesicles, which contained granular material, were seen in this basal region (Figure 4.28). Tonofilaments, and in some cases glycogen, attached to the outer membranes of the vesicles.

The ultrastructural morphology of the skin periderm resembled that of the electron-lucent horn bud periderm cells. The basal plasma membrane of the electron-lucent periderm cells in the horn bud had short villous processes which interlocked with similar structures from underlying intermediate cells. Short

desmosomes linked the periderm and intermediate cells.

Dermis

As reported in the light microscopy section a combination of mesenchymal cells and fibroblasts were found in horn bud papillary dermis, whilst mesenchymal cells dominated the skin papillary dermis.

The mesenchymal cells in both the horn bud and skin were stellate in shape with large, oval nuclei. The cytoplasm was drawn out into several long processes which made contact with neighbouring cells (Figure 4.29). The areas of contact between the cells were marked by a localized increase in density which resembled desmosomes. The mesenchymal cytoplasm contained small amounts of rough endoplasmic reticulum, a Golgi complex and mitochondria. A few cytofilaments and polyribosomes were also present (Figure 4.30).

Fibroblasts containing elongated nuclei were seen scattered throughout the dermis. The abundant cytoplasm was drawn out into a long cytoplasmic process which contained an intricate formation of rough endoplasmic reticulum (Figure 4.31).

Cells assumed to be mast cell precursors were present in the papillary dermis. These cells had round nuclei enclosed in abundant cytoplasm which contained numerous small vesicles and electron-dense bodies (Figure 4.32). In some cases small granules were found within the electron-dense bodies. Distended rough endoplasmic reticulum, mitochondria and polyribosomes were also observed in the cell. It is possible that these cells corresponded to the small, dark basophilic cells seen with the light microscope.

104-108 DAYS OF GESTATIONStratum basale

Two to four layers of columnar cells formed the stratum basale in the horn bud region. The nuclei of these cells were located either in the middle or basal regions of the cells. The nuclei were enclosed in electron-dense cytoplasm which contained many mitochondria, elongated rough endoplasmic reticulum profiles, a few clumps of vesicles and numerous free ribosomes. Several mitotic figures were observed in the basale layer.

In the skin surrounding the horn bud a single layer of cuboidal cells formed the stratum basale. The nuclei of these keratinocytes were oblong in shape. The cytoplasm was electron-dense with numerous mitochondria and free ribosomes. A few rough endoplasmic reticular profiles were also observed in these cells.

Several melanocytes were seen within the horn bud stratum basale, as well as in the dermis adjacent to the basale cells. The melanocytes within the stratum basale were attached to adjacent keratinocytes by a few desmosomes. Round nuclei surrounded by electron-lucent cytoplasm typified the melanocytes seen in this age group. The cytoplasm contained electron-dense melanosomes, in addition to polyribosomes and clear vesicles (Figure 4.33). The melanocytes possessed several cytoplasmic processes which extended between neighbouring basale cells. Melanocytes were occasionally observed in the skin of the forehead. The morphology of the electron-dense bodies of these cells was similar to the horn bud melanocytes.

Stratum intermedium

The superficial intermedium cell layers in the horn bud contained large, round cells which were filled with evenly distributed glycogen granules and

tonofilaments. The cells contained apically located nuclei which were enclosed in a thin rim of electron-dense cytoplasm. The deeper stratum intermedium cell layers were composed of smaller cells which had features of both intermediate and basale cells. The basally located nuclei of these cells were surrounded by electron-dense cytoplasm which contained numerous mitochondria, glycogen granules, free ribosomes and several thick tonofilament bundles. The cells in this transitional layer were loosely bound by short desmosomes.

Between the typical stratum intermedium cells, in the horn bud, were dendritic, electron-lucent cells with several mitochondria, a small amount of rough endoplasmic reticulum, and numerous small electron-dense bodies, some of which were rod-shaped (Figure 4.34). These cells were identified as Langerhans cells due to the presence of small electron-dense granules and cytoplasmic processes which extended between neighbouring intermediate cells. A few desmosomes were observed between the Langerhans cells and the intermediate cells. One of the Langerhans cells observed in the present study contained a large membrane-bound area which contained densely-packed glycogen granules. The glycogen appeared to have been engulfed from a neighbouring intermedium cell. However it was noted that the glycogen granules in the Langerhans cell were more closely packed compared to the glycogen granules in adjacent intermedium cells. The Langerhans cells identified are thought to correspond to the small intensely eosinophilic cells identified in the Light Microscopy section.

In the forehead skin four cell layers formed the stratum intermedium. The cells had a similar morphology to the superficial intermediate layers of the horn bud.

Periderm

The apical surface of the periderm cells, in the horn bud region, possessed long and short microvilli, some of which were branched (Figure 4.35). The shorter microvilli were diffusely distributed, whereas the longer microvilli were arranged in small tufts. The microvilli were filled with filamentous bundles which also occurred below the surface membrane. The "fuzzy" coat seen covering the periderm surface of the horn bud in younger foetuses was absent.

Because of the flattened nature of the horn bud periderm cells, nuclei were absent in most sections. The periderm cytoplasm contained a dense mesh of tonofilaments, with a few glycogen granules and isolated vesicles. The tonofilaments were evenly distributed throughout the cell and did not appear in bundles as seen in earlier foetuses. Closely spaced desmosomes linked adjacent periderm cells.

Protrusions were seen arising from periderm cells in the groove surrounding the horn bud (Figure 4.36). These protrusions were attached to the main part of the underlying periderm cells by broad pedicles. The protrusions tended to occur at the junction of two periderm cells and involved the cytoplasm of both cells. The contents of the protrusions were similar to the contents of the underlying periderm cells, except for the presence of a few large electron-dense bodies in some of the protrusions.

The skin surrounding the circular groove was covered by spindle-shaped periderm which lacked protrusions. As in the horn bud these periderm cells were studded with microvilli of varying lengths. The skin periderm cells differed from those covering the horn bud in that they contained many vesicles directly below the surface.

Dermis

As noted with the Light Microscopy section the papillary dermis in the horn bud contained a mixture of fibroblasts and mesenchymal cells. Wide areas containing a few collagen fibres separated the cells. The fibroblasts were more mature with a larger amount of rough endoplasmic reticulum and more cytofilaments than in younger animals (Figure 4.37). At this stage dilated rough endoplasmic reticular profiles contained vesicles of various sizes, besides the granular material observed in the 71-day-old foetus. The shorter segments of rough endoplasmic reticulum contained only granular material with no vesicles being observed.

The dermis of the adjacent skin contained closely packed undifferentiated mesenchymal cells many of which contained small, round to irregular-shaped nuclei surrounded by electron-dense cytoplasm (Figure 4.38 A). Also present were maturing mesenchymal cells which contained less electron-dense oval or irregular-shaped nuclei and large amounts of rough endoplasmic reticulum. The undifferentiated and maturing mesenchymal cells were in contact with each other over wide areas of their surface (Figure 4.38 B).

112-117 DAYS OF GESTATIONStratum basale

Stratum basale cells in the horn bud region contained large nuclei which were enclosed in a thin rim of electron-dense cytoplasm. Numerous mitochondria and ribosomes were seen in the cytoplasm (Figure 4.39). Hemidesmosomes were present between the basal plasma membrane and the basal lamina.

Melanocytes with a morphology similar to those described in the 104 to

108 age group were observed within the stratum basale.

Stratum intermedium

As in previous foetuses the superficial intermedium cells in the horn bud were large with apically positioned nuclei. A few ribosomes and small electron-dense bodies were present in the perinuclear cytoplasm. The rest of the cytoplasm was occupied by glycogen granules and tonofilament bundles. The glycogen granules were arranged into a reticular pattern. Deeper intermedium layers in the horn bud contained cells with an ultrastructural morphology similar to that of the stratum basale cells. However, these stratum intermedium cells had large cellular volumes and glycogen granules, in addition to the numerous mitochondria and ribosomes observed in the stratum basale cells.

Periderm

Two layers of periderm cells were present in some areas of the horn bud. The superficial periderm layer had a dense covering of microvilli, most of which were arranged in groups. Thick tonofilaments were present below the plasma membrane in areas where the microvilli occurred. Loose tonofilaments were observed throughout the cytoplasm, but were more numerous in the perinuclear area. Apart from tonofilaments the cytoplasm of both periderm layers contained numerous glycogen granules and round electron-dense bodies of various sizes. The junction between the two periderm cell layers was marked by the presence of long interdigitating processes linked by numerous desmosomes (Figure 4.40).

The periderm cells in the groove surrounding the horn bud had several protrusions which arose from the central and marginal areas of the cell (Figure 4.41). The surface membrane covering the protrusions was indistinct in most cases, although intact microvilli were occasionally seen.

Dermis

A combination of electron-dense fibroblasts and more electron-lucent mesenchymal cells were observed in both the horn bud and the skin. The mesenchymal cells were as described in previous foetuses. The fibroblasts contained elongated nuclei surrounded by cytoplasm which was filled with ribosomes, cytofilaments and a few mitochondria. In addition to the mesenchymal cells and fibroblasts were intermediate cell types which had features of both mesenchymal cells and fibroblasts (Figure 4.39). The electron-lucent cytoplasm of these intermediate cells contained numerous ribosomes, and dilated rough endoplasmic reticulum with small electron-dense vesicles.

122-125 DAYS OF GESTATION

Stratum basale

In the horn bud columnar and cuboidal cells formed this layer. The cells were roughly arranged in a row. In many of the cells oblong-shaped nuclei were located in the wide apical regions, whilst numerous round mitochondria were positioned in the narrow basal zone. The cytoplasm was electron-dense due to the presence of numerous free ribosomes and rough endoplasmic reticulum. The basale cells in the horn bud had long lateral processes which interdigitated with bordering cells. However, few desmosomes were observed between adjacent cells which resulted in the existence of wide intercellular spaces. Numerous desmosomes occurred between the basale and intermediate cell layers in the horn bud. The basale membrane had several infoldings which corresponded to similar structures on the basal lamina.

In the skin the stratum basale was composed of regularly arranged low columnar cells with large oblong nuclei. Numerous desmosomes connected the neighbouring cells resulting in very narrow intercellular spaces. The infolding of

the basal membrane observed in the horn bud stratum basale was not seen in the skin basal lamina.

Stratum intermedium

Ultrastructurally two types of intermedium cells were recognized. The first type was similar to those found in the superficial layers of the stratum intermedium in 112 to 117 day old bovine foetuses. These cells were filled with glycogen granules and tonofilaments, both of which were arranged in a lattice throughout the cell. The second type of intermedium cell lacked glycogen and tonofilaments in the perinuclear area (Figure 4.42). These cells corresponded to the intermedium cells from the central regions of the horn bud which were described in the Scanning Electron Microscopy section.

Periderm

The periderm in the horn bud consisted of a single discontinuous layer. Few microvilli were recognized on the periderm surface which was marked by several irregular indentations. The central regions of the periderm cells were filled with tonofilament bundles. Several irregular-shaped protrusions arose from the periderm surface in groove surrounding the horn bud. However, fewer protrusions were seen in this age group compared to the protrusions seen in the 112 to 117 age group. Many cavities were observed in the groove.

In the skin the periderm cells were flat with thin overlapping edges. A few microvilli were present, but no protrusions or infoldings were observed.

Dermis

Cells in the horn bud were sparsely distributed. Fibroblasts with elongated nuclei were frequently observed in all regions of the dermis. The

fibroblast cytoplasm at this stage contained numerous cytofilaments which partially obscured the cellular organelles. A few mitochondria, rough endoplasmic reticulum and small vesicles which contained moderately electron-dense material were present in the fibroblast cytoplasm.

The skin surrounding the horn bud was composed of numerous mesenchymal cells, most of which made intercellular contact with surrounding cells.

182-206 DAYS OF GESTATION

Stratum basale

The stratum basale in the horn bud was characterized by cells with large nuclei and scant cytoplasm. The cytoplasm contained a large amount of rough endoplasmic reticulum and vesicles, particularly in the basal regions of the cells. Mitochondria were scattered randomly throughout the cytoplasm. A few desmosomes connected the adjacent basale cells.

The stratum basale of the adjacent forehead skin had oval to oblong nuclei. The scant cytoplasm contained large amounts of glycogen and a few small mitochondria. Numerous desmosomes were present between adjacent basale cells, as well as between basale and spinosum cells. Thick tonofilaments were attached to the desmosomal plaques.

Stratum intermedium

The stratum spinosum was composed of cells with round nuclei, several round electron-dense bodies and numerous tonofilaments. The spinosal layer directly below the stratum granulosum contained few glycogen granules, whereas the lower spinosal layers still contained moderate quantities of

glycogen.

Below the stratum corneum was a granular layer formed by degenerating cells (Figure 4.43). The pyknotic nuclei were enclosed in cytoplasm which contained several electron-dense keratohyalin granules, a few mitochondria and peripherally located tonofilament bundles. Desmosomes connecting the stratum granulosum cells to the stratum corneum were still distinct.

Periderm

By 182 days of gestation most of the periderm had sloughed off exposing the underlying stratum corneum.

Dermis

Mature fibroblasts were observed in the horn bud and skin. The fibroblasts has elongated nuclei surrounded by abundant electron-dense cytoplasm. The cytoplasm was drawn out into a long process which contained the bulk of the cellular organelles. Extensive, well-developed rough endoplasmic reticulum, polyribosomes and mitochondria were observed in the cytoplasm (Figure 4.44).

Mast cells were noted close to the basal lamina in the papillary dermis. These were rounded cells with large dark nuclei and several long cytoplasmic processes (Figure 4.45). Numerous electron-dense bodies were located close to the plasma membrane. A few electron-dense bodies contained dense bands of material. Large mitochondria, small vesicles and granules were scattered throughout the cytoplasm.

POST-NATAL DEVELOPMENT IN CATTLE

Post-natally no cytological differences were noted between epidermal cells of the horn bud and those of the skin.

STRATUM BASALE

The stratum basale was formed by a single row of low columnar cells with large oblong nuclei and scant cytoplasm. The cytoplasm contained numerous polyribosomes, mitochondria and basally-located rough endoplasmic reticulum. A few small vesicles containing moderately electron-dense material were observed in the basal regions of the cells. Occasional lipid droplets were found in a few basale cells. The outer limits of the basale cells contained thick tonofilament bundles, which extended into the cytoplasmic processes which extended from the basal plasma membrane (Figure 4.46).

Several melanocytes were scattered throughout the basale layer. The melanocytes were identified by the presence of round electron-dense melanosomes and numerous vacuoles (Figure 4.47). No desmosomal links were seen between the melanocytes and the surrounding keratinocytes.

STRATUM SPINOSUM

The stratum spinosum was composed of cells with oval shaped nuclei which were surrounded by a large amount of cytoplasm. The cytoplasm was dominated by tonofilament bundles which radiated outwards to attach to desmosomal plaques (Figure 4.48). In addition to the tonofilaments the cytoplasm contained groups of mitochondria, rough endoplasmic reticulum and ribosomes.

STRATUM GRANULOSUM

The stratum granulosum was characterized by the presence of large irregular-shaped keratohyalin granules (Figure 4.49). In some cases the keratohyalin granules were positioned close to the nucleus. In the deeper granulosum cells, the nuclei and cellular organelles were still intact, whereas in the more superficial layers the cells had began to degenerate. These degenerating cells lacked distinct cell borders and contained fragmented cellular organelles (Figure 4.50).

DISCUSSION

PRE-NATAL HORN BUD DEVELOPMENT

STRATUM BASALE

In the bovine foetus between 71 and 206 days of gestation the number of cell layers forming the horn bud stratum basale varied between one and four. The greatest number of cell layers was seen at 104-days of gestation. In the horn bud the formation of a multi-layered stratum basale was due to rapid cell division in this region, coupled with a slower differentiation of basale cells into intermedium cells. In developing skin in the human (Breathnach and Robins, 1969; Foster *et al.*, 1988), pig (Meyer *et al.*, 1986) and mouse (Du Brul, 1972) the stratum basale is maintained as a single layer throughout development.

Wide intercellular spaces, in some cases containing glycogen, were noted between the horn bud basale cells in the current study. The wide spaces occurred in areas where few desmosomes were present. The presence of wide intercellular areas, filled with glycogen, has been noted in the skin of human foetuses between 12 and 22 days of gestation (Hashimoto *et al.*, 1966^b).

However, in the present study intercellular glycogen was found only in the 71-day-old foetus. In addition only small amounts of intracellular glycogen were noted in the horn bud basale cells using histochemical and ultrastructural techniques.

STRATUM INTERMEDIUM

In the present study the stratum intermedium in the horn bud of bovine foetuses was seen to consist of between three and twenty-six cell layers. Rapid-cell division of the stratum basale resulted in a thick epidermis at 125 days of gestation. After 159 days the number of layers forming the intermediate layer decreased, until only three layers were present at 206 days of gestation. The increase and subsequent decrease in the number of cell layers forming the stratum intermedium has been noted in bovine (Lyne and Hollis, 1960; Lyne and Heideman, 1959), sheep (Lyne, 1957; Lyne and Hollis, 1968), human (Foster *et al.*, 1988), rat (Bauer, 1972), and mouse foetuses (Rimmer, 1968). In the majority of bovine foetuses in the current study, the horn bud epidermis was thicker than that of the adjacent skin. Similar observations have been made in sheep, where the horn bud epidermis was significantly thicker than the skin in foetuses between 75 and 88 days of gestation (Lyne and Hollis, 1973). The subsequent decrease in epidermal thickness coincided with the cessation of hair follicle initiation in the sheep horn bud (Lyne and Hollis, 1973). This observation is supported by results obtained in the current study, in which the decrease in epidermal thickness of the horn bud centre coincided with a decrease in the presence of new hair germs, as well as the formation of hair canals. However, although the horn bud centre had a smaller number of cell layers, active cell proliferation was occurring at the horn bud margins, where as many as twenty-six cell layers were seen.

The ultrastructural morphology of the horn bud stratum intermedium cells was similar to that of intermedium cells in the human (Whittaker and Adams, 1971; Holbrook and Odland, 1975), rabbit (Maruyama *et al.*, 1988) and rat (Bauer, 1972). The stratum intermedium cells had a large cell volume, most of which was occupied by glycogen granules. The nuclei usually occupied an apical position in the cell. In the current study tonofilaments were identified ultrastructurally in the intermedium cell cytoplasm as early as 71 days of gestation. As the foetus matured the concentration of tonofilament bundles increased until they formed a thick lattice, as seen in the scanning electron micrograph of a 125-day-old foetus. The presence of tonofilaments has been reported pre-natally in the human (Breathnach, 1971^a) and pig (Meyer *et al.*, 1986). The tonofilaments are thought to act as a cytoskeleton which stabilizes the structure of the cell (Meyer *et al.*, 1986).

In the current study Langerhans cells were identified in the horn bud stratum intermedium of a 104-day-old foetus at both light and transmission electron microscopic levels. The dendritic Langerhans cells lacked tonofilaments and well-developed desmosomes, but contained small, round electron-dense granules. The ultrastructural morphology of the Langerhans cells was similar to that of Langerhans cells identified in human foetuses (Breathnach and Wyllie, 1965; Breathnach, 1971^{a,b}). Langerhans cells are thought to function as intraepidermal phagocytes (Zelickson, 1965). In the 104-day-old bovine foetus a membrane-bound glycogen-filled area was observed in one of the Langerhans cells. Breathnach (1971) has reported similar occurrences in human foetuses, where Langerhans cells engulf sections of disintegrating keratinocyte cytoplasm.

PERIDERM

At 71 days of gestation a layer of flakes, probably originating from the periderm cells, covered the horn bud. In addition, transmission electron micrographs of the periderm at this stage revealed the presence of a "fuzzy" electron-dense layer covering the horn bud. The presence of a mucopolysaccharide layer covering the periderm in human foetuses has been described by Hoyes (1967). Furthermore, Holbrook and Odland (1975) noted the presence of a "fuzzy coat" covering the periderm in human foetuses between 55 and 75 days of gestation. They believed that the presence of this layer of mucopolysaccharides was evidence for the role of the periderm in the secretion of substances. However it has also been suggested that the periderm is involved in the absorption of substances from the amniotic fluid (Breathnach, 1971^b). The presence of microvilli increases the surface area of the periderm in contact with amniotic fluid, thus facilitating the absorption of substances such as glucose (Breathnach, 1971^b). In the current study, microvilli covered the periderm of both the horn bud and skin. The microvillous nature of the horn bud periderm is similar to that of the skin periderm in the human (Breathnach, 1971^a; Meller *et al.*, 1973), pig (Meyer *et al.*, 1986), rabbit (Maruyama *et al.*, 1988) and mouse (M'Boneko and Merker, 1988). In the present study, an increase in the density of microvilli with age was noted. This has also been observed in foetal pigs, where the density of microvilli increased until 75 days of gestation (Meyer *et al.*, 1986). In addition to microvilli, numerous pinocytotic vesicles were observed in the horn bud region at 71 days of gestation. The presence of pinocytotic vesicles supports the theory that the periderm is involved in the uptake of substances from the amniotic fluid.

In the present study, large amounts of glycogen were present in the horn bud periderm between 71 and 125 days of gestation, after which the amounts

steadily decreased. Verma *et al.* (1976) noted large amounts of glycogen and alkaline phosphatase in periderm cells in the human foetus. They suggested that alkaline phosphatase facilitated the absorption of glucose into the periderm where it was stored as glycogen. The glycogen in developing epidermis serves as a rapid source of energy for cell division, keratinization and the production of secretions (Serri *et al.*, 1962).

In the current study, a scanning electron microscopic examination of the horn bud between 104 and 125 days of gestation revealed the presence of numerous protrusions in the groove surrounding the horn bud. Similar protrusions have been described in the skin of human foetuses between 65 and 120 days of gestation (Holbrook and Odland, 1975, 1980; Whittaker and Adams, 1971). The peridermal protrusions observed in the present study contained numerous tonofilaments and a few large electron-dense bodies. In human foetuses the protrusions contained a large amount of glycogen which was subdivided into compartments by tonofilament bundles (Holbrook and Odland, 1975; Breathnach, 1971^a). Furthermore, in human foetuses the protrusions arose from the central regions of the periderm cells, whereas in the present study the protrusions usually formed along the periderm cell margins. A study of abdominal skin development in bovine foetuses, between 68 and 183 days of gestation, did not report the presence of peridermal protrusions (Lyne and Heideman, 1959). This suggests that in bovine foetuses the occurrence of protrusions is restricted to the horn bud region. Polakowska *et al.* (1994) have shown that the formation of protrusions in human foetal skin coincides with the appearance of apoptotic markers and DNA fragmentation, both indicators of cell degeneration. This is in agreement with findings in the current study, in which periderm cells were shed soon after the formation of protrusions. The loss of periderm cells resulted in the formation of cavities on the horn bud surface, as well as in the groove surrounding the horn bud.

In the 71-day-old foetus, vesicles containing small electron-dense bodies were observed near the periderm-intermedium junction in the horn bud. Vesicles positioned close to cellular margins have been observed in foetal rat skin between 17 and 19 days of gestation (Hayward and Kent, 1983). In the rat these vesicles were thought to be annular gap junctions which contained cytoplasm (Hayward and Kent, 1983). However, in the present study the vesicles were probably not annular gap junctions as the contents of the vesicles were not the same as the surrounding cytoplasm. The small electron-dense bodies seen within the vesicles were not observed in the periderm cytoplasm.

DERMIS

In the present study a large number of mesenchymal cells, fibroblasts, and blood vessels were observed below the horn bud epidermis at 71 days of gestation. This contrasted with the adjacent skin where sparsely distributed cells were seen. The presence of large numbers of cells and blood vessels below developing skin was interpreted by Maruyama *et al.* (1988) as evidence of epidermal-dermal interactions. They suggested that the epidermal-dermal interactions caused rapid epidermal proliferation. This was not the case in the present study where the horn bud and skin contained a similar number of epidermal cell layers despite the high cellularity and vascularity below the horn bud. No morphological evidence of an epidermal-dermal interaction was found in the present study. However, it is known that such interactions are responsible for the formation of a variety of ectodermal derivatives such as feathers (Cairns and Saunders, 1954; Zwilling, 1956; McLoughlin, 1961) and glands (Grobstein, 1953; Auerbach, 1960).

The ultrastructural morphology of the mesenchymal cells observed in the present study is similar to that of mesenchymal cells in the human (Breathnach, 1971^a, 1978) and mouse (Van Exan and Hardy, 1984). The mesenchymal cells in the present study were stellate in shape, with long cytoplasmic processes and a small amount of rough endoplasmic reticulum. A predominance of dark mesenchymal cells was noted at 104 days of gestation in the skin surrounding the horn bud. The presence of mesenchymal varying in electron density has also been noted in the human foetus between 6 and 14 days of gestation. In the current study cell contacts between adjacent mesenchymal cells were common. The structure of these cells corresponds to that of undifferentiated mesenchymal cells observed in the mouse between 12 and 13 days of gestation (Van Exan and Hardy, 1984), as well as "type 1" cells observed in the 8-week-old human foetus (Breathnach, 1978). As the mesenchymal cells in the horn bud and adjacent skin differentiated into immature fibroblasts, the desmosomal-like contacts between the cells were lost. In addition, the amount of rough endoplasmic reticulum and the density of cytofilaments within the cells increased, indicating the commencement of collagen production. Similar immature fibroblasts have been observed in the human foetus between 14 and 21 weeks of gestation (Breathnach, 1978).

In the current study, cells assumed to be mast cell precursors were seen in the 71 day old foetus. These presumptive mast cell precursors contained round nuclei, a few round electron-dense bodies and numerous clear vesicles. Similar cells, thought to be mast cell precursors, have been observed in 14-week-old human foetuses (Breathnach, 1978). In the present observations mast cells were positively identified in the 198-day-old foetus. The mast cells contained characteristic electron-dense bodies, as well as numerous cytoplasmic processes.

POST-NATAL HORN BUD DEVELOPMENT

The components of the horn bud epidermis in the calves and kids were in most cases identical to those of skin in the human (Brody, 1960), adult cattle (Goldsberry and Calhoun., 1959; Lloyd *et al.*, 1979), sheep (Kozlowski and Calhoun, 1969) and dog (Creed, 1958). Ultrastructurally the strata basale and spinosum in all the calves studied contained tonofilament bundles which attached to desmosomal plaques. The presence of tonofilaments post-natally has been reported in the skin of the human (Breathnach, 1971^a), dog (Webb and Calhoun, 1954) and marsupial native cat (Pralomkran *et al.*, 1990)

The development of horn tubules in the calves and kids was in close agreement with observations made by Lyne and Hollis (1973) in Merino sheep. In the current study, as in the sheep studied by Lyne and Hollis (1973), the horn tubules were formed by pale-staining cells located over the tips of dermal papillae. In the present study horn-tubule formation was more advanced in kids, with distinct tubular and intertubular regions being observed in the 3-week-old animals.

Apart from keratinocytes, a few melanocytes and Merkel cells were observed in the horn bud stratum basale. The ultrastructural morphology of the melanocytes was in agreement with observations in the human (Hashimoto *et al.*, 1966^a; Breathnach, 1980) and opossum (Lyne *et al.*, 1970). The melanocytes in the current study contained vacuolated and partially degraded mitochondria. Similar cells have been observed by Breathnach (1980), who has suggested that the mitochondria in melanocytes are more susceptible to degenerative changes compared to mitochondria in keratinocytes. However, Hashimoto (1971) observed a combination of degenerative and intact melanocytes in 12-week-old human foetuses. He stated that the presence of a few degenerating melanocytes within the epidermis was a normal occurrence and was not caused

by post-mortem autolysis.

SUMMARY

STRATUM BASALE

1. The stratum basale was composed of between one and four layers of cuboidal to columnar cells with large nuclei and scant basophilic cytoplasm.
2. Wide intercellular spaces, sometimes containing glycogen, separated the basale cells in young foetuses. In addition to the intercellular glycogen intracellular glycogen was demonstrated using histochemical and ultrastructural techniques.

STRATUM INTERMEDIUM

1. The stratum intermedium was characterized by large, round cells with apically placed nuclei.
2. Histochemistry and transmission electron microscopy demonstrated the presence of basally located glycogen in the intermedium cells.
3. In addition to glycogen the cells contained tonofilaments which were arranged in a reticular pattern throughout most of the cell.
4. Cells assumed to be Langerhans cells were identified with the light microscope and transmission electron microscope at 104 days of gestation.

5. By 159 days of gestation the stratum intermedium had differentiated into corneum, granulosum and spinosum cell layers.

PERIDERM

1. The periderm cells in the horn bud region initially had large central regions surrounded by flattened lateral regions. Later the cells became irregular in shape with long tapered basal processes.
2. Histochemistry and electron microscopy revealed the presence of large amounts of glycogen in most of the periderm cells in the horn bud region.
3. Protrusions in the groove surrounding the horn base were first seen at 104 days of gestation. By 125 days of gestation the periderm cells possessing protrusions had begun to slough leaving deep cavities.
4. The desquamation of the periderm coincided with the development of an underlying corneum layer. The periderm in the horn bud region was lost by 182 days of gestation.

DERMIS

1. Mesenchymal cells were stellate-shaped with large nuclei and a small amount of cytoplasm which was drawn out into slender processes. A mixture of mesenchymal cells with dark and pale-stained nuclei were identified.
2. Immature fibroblasts with characteristics of both mesenchymal cells and

mature fibroblasts were identified using the transmission electron microscope.

3. Cells assumed to be mast cell precursors were observed at 71 days of gestation. Ultrastructurally the cells contained a few round electron-dense bodies enclosed in abundant cytoplasm.

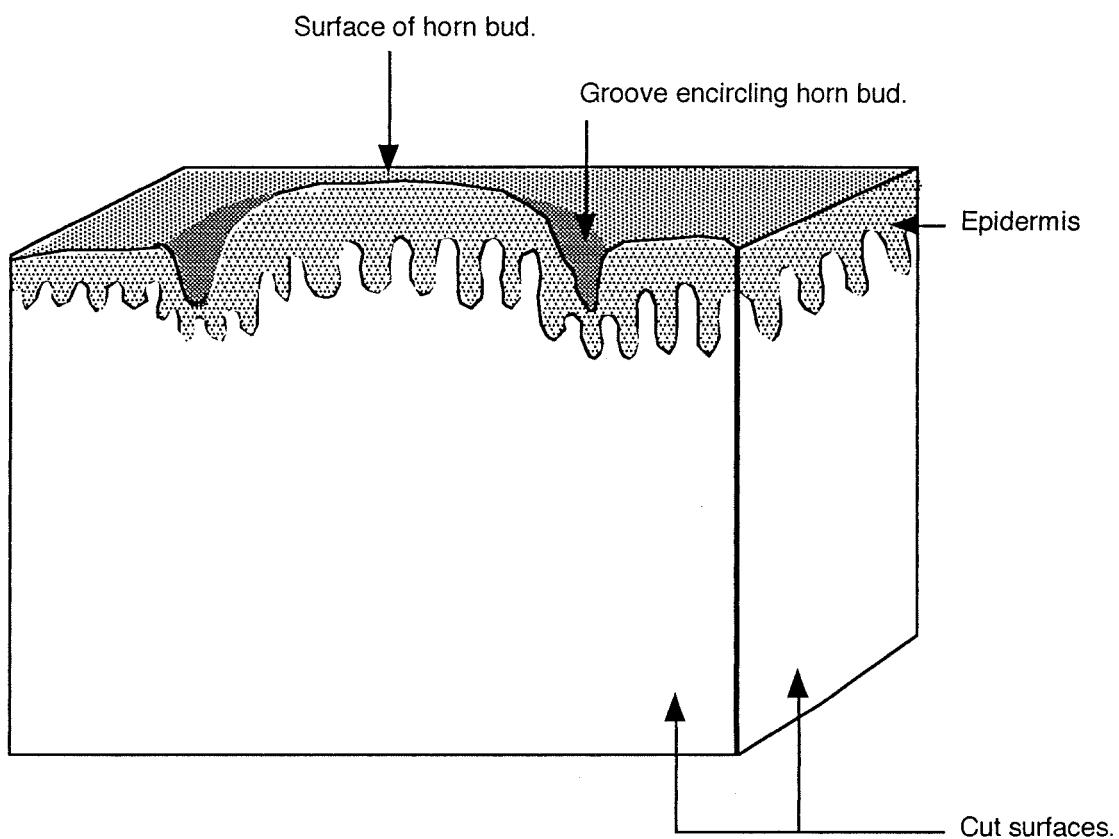


Figure 4.1 Graphic representation of the surfaces viewed with the scanning electron microscope.

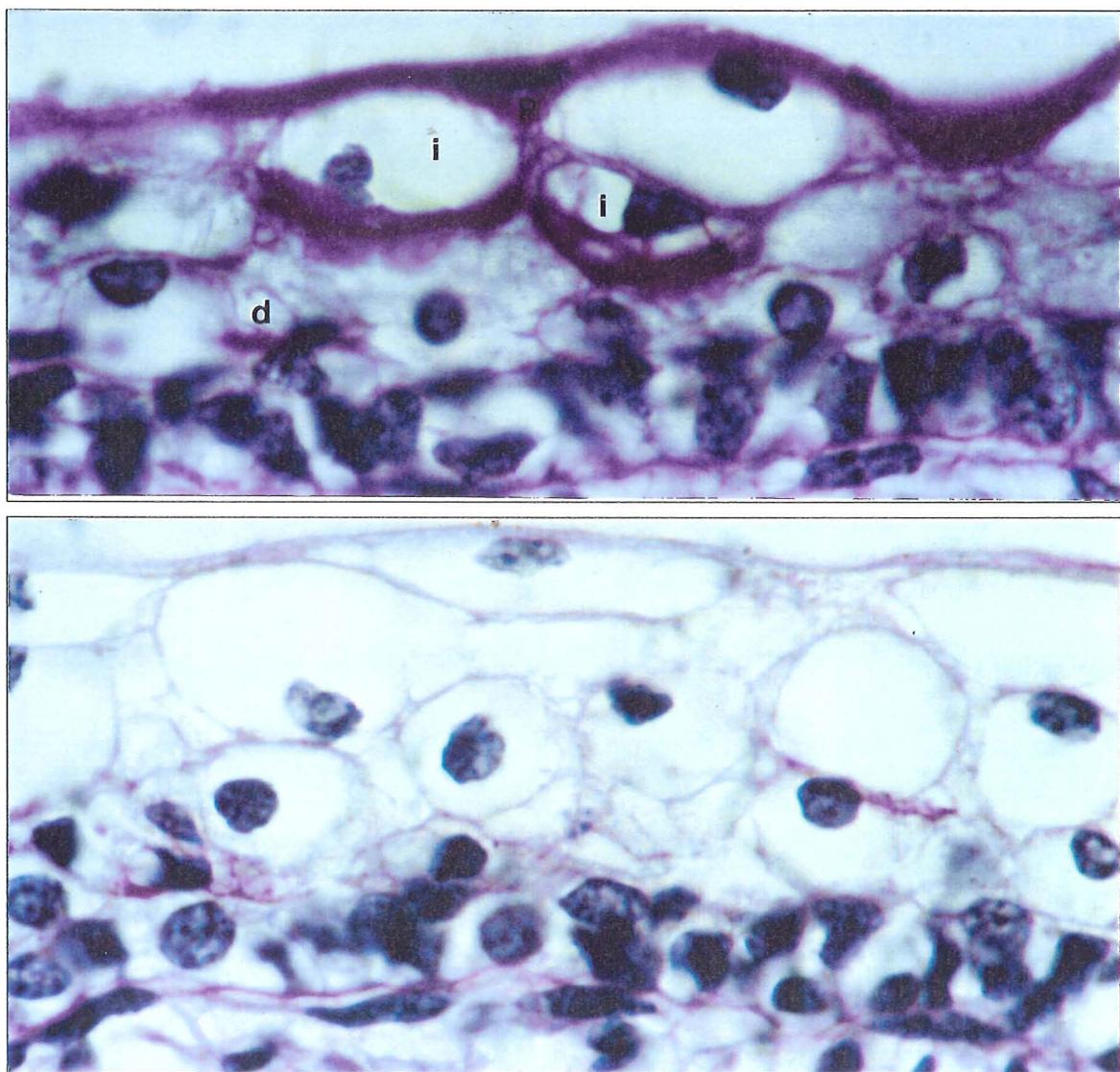


Figure 4.2 · 71-day-old bovine foetus. **A.** The periderm (P) in the horn bud region has stained intensely for the presence of glycogen. Glycogen is concentrated in the basal regions of the superficial layers of the stratum intermedium (i). A few localized areas of glycogen are seen in the deep intermedium cell layer (d). Periodic-Acid-Schiff. X 1,875.

B. Traces of glycogen are seen in the skin surrounding the horn. Periodic-Acid-Schiff. X 1,875.

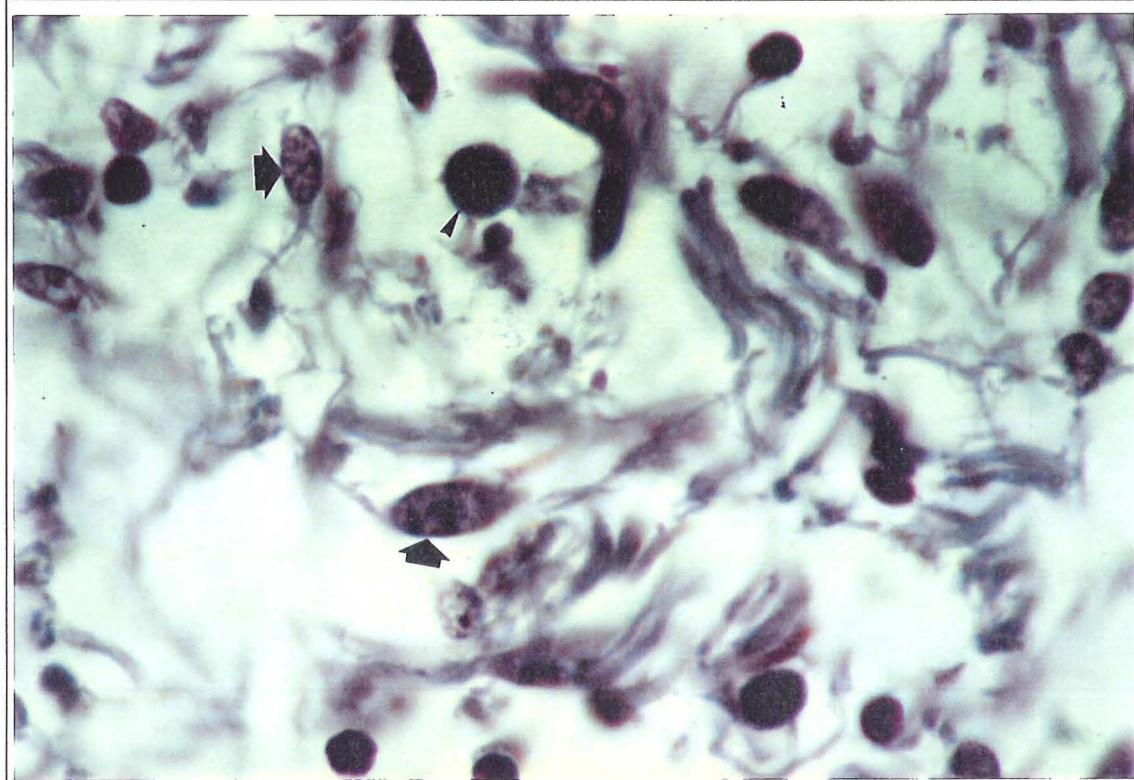


Figure 4.3 71-day-old bovine foetus. A mixture of cells is present in the deeper regions of the horn bud papillary dermis. Amongst the cells are mesenchymal cells (arrows) with large oval-shaped nuclei and scant basophilic cytoplasm which is drawn out into several long processes. In addition presumptive mast cells with dark round nuclei and basophilic cytoplasm are observed (arrowhead). Masson's trichrome. X 1,875.

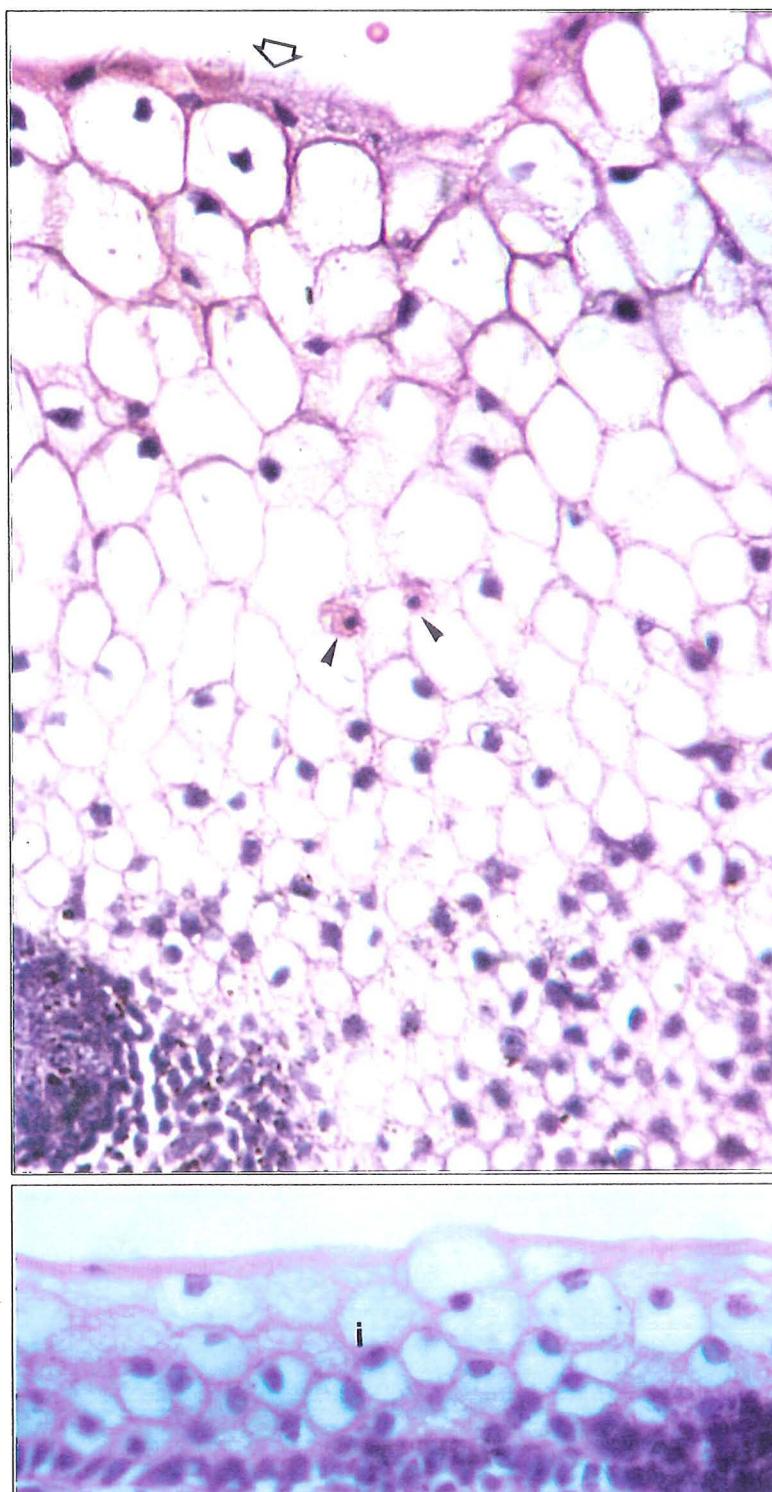


Figure 4.4

A. The horn bud region is formed by an epidermis twelve to fourteen cell layers thick. The stratum intermedium cells decrease in size towards the stratum basale. Most of the cells have pale-staining cytoplasm, but occasional presumptive Langerhans cells with intensely eosinophilic cytoplasm (arrowheads) are also seen. A brush border (arrow) is visible on the periderm surface. H&E. X 750.

B. The skin surrounding the horn bud has three to four intermedium cell layers (i) underlying the periderm. H&E. X 750.

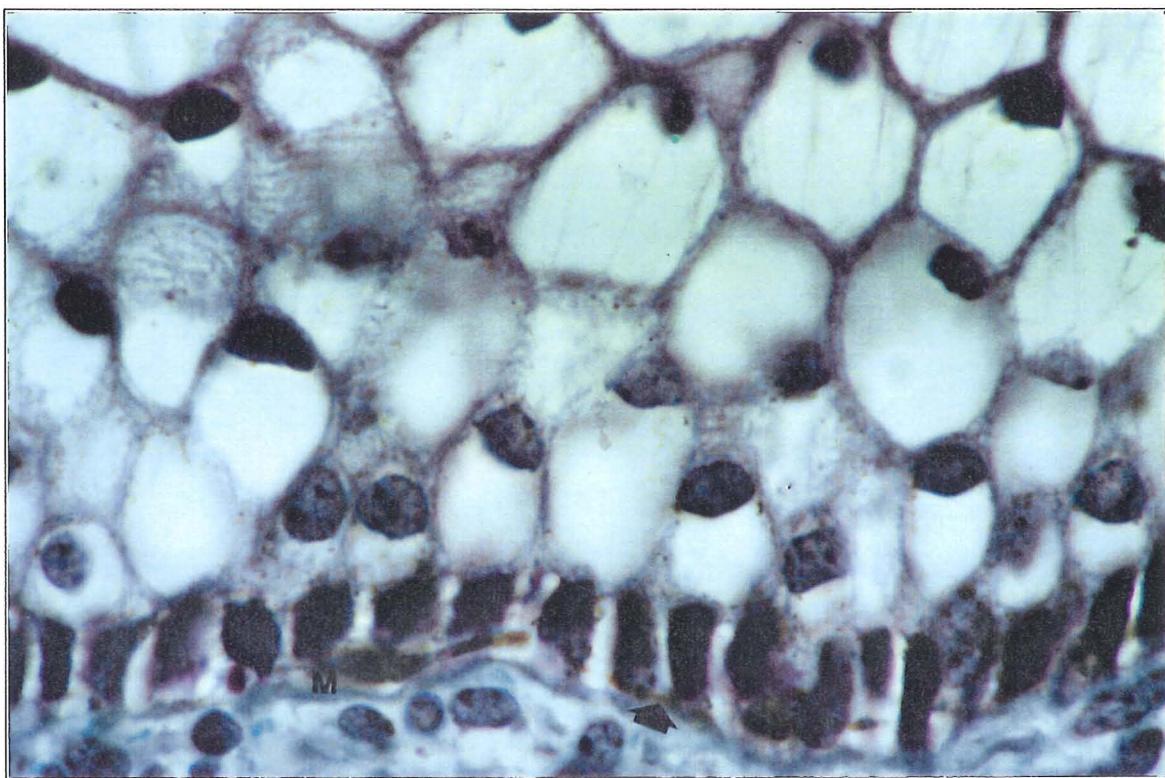


Figure 4.5 117-day-old bovine foetus. A melanocyte (m) with long cytoplasmic processes (arrow) is seen below the stratum basale. Masson's trichrome. X 1,875.



Figure 4.6 117-day-old bovine foetus. Two intensely PAS-positive periderm layers (P) are seen above the stratum intermedium (I). The glycogen within the stratum intermedium is arranged in a reticular pattern. Periodic-Acid-Schiff. X 750.

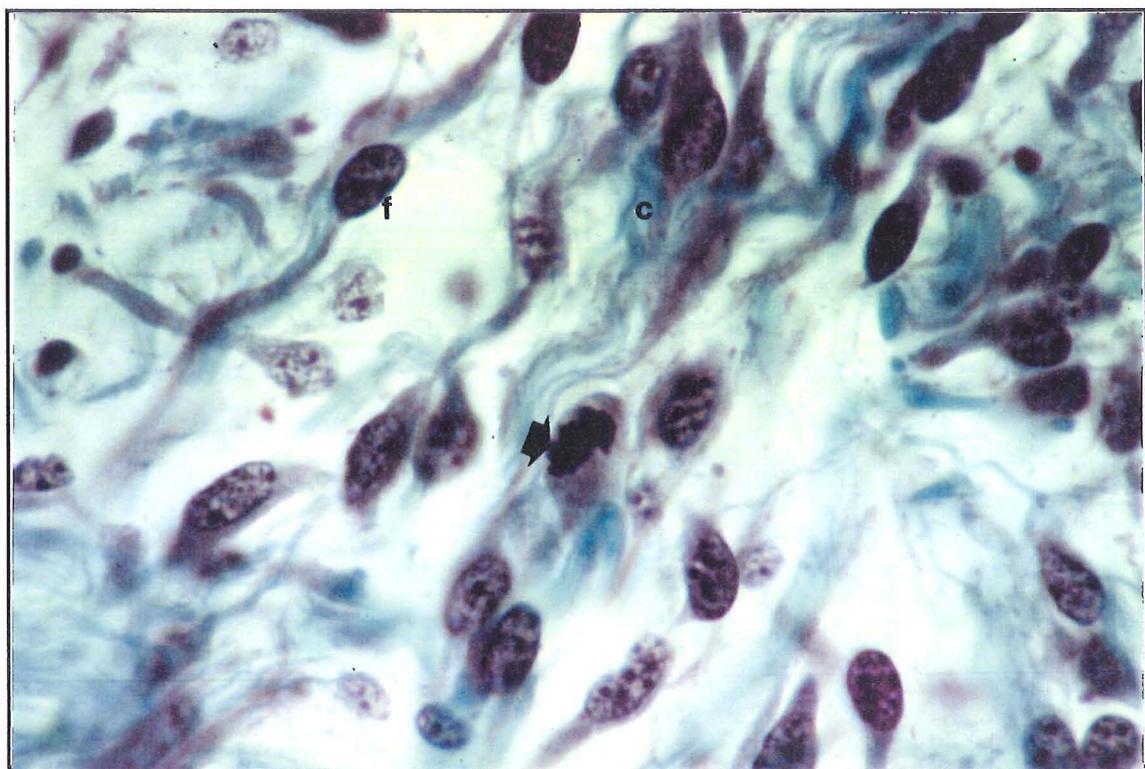


Figure 4.7 112-day-old bovine foetus. The reticular dermis is composed of collagen bundles (c) lined with fibroblasts (f), which contain oval to elongated nuclei and basophilic cytoplasm. A mitotic figure (arrow) is seen in a cell assumed to be a fibroblast. Masson's trichrome. X 1,875.



Figure 4.8 146-day-old bovine foetus. Irregular-shaped cells form the horn bud periderm (P) which is absent in some areas (arrow). Masson's trichrome. X 750.

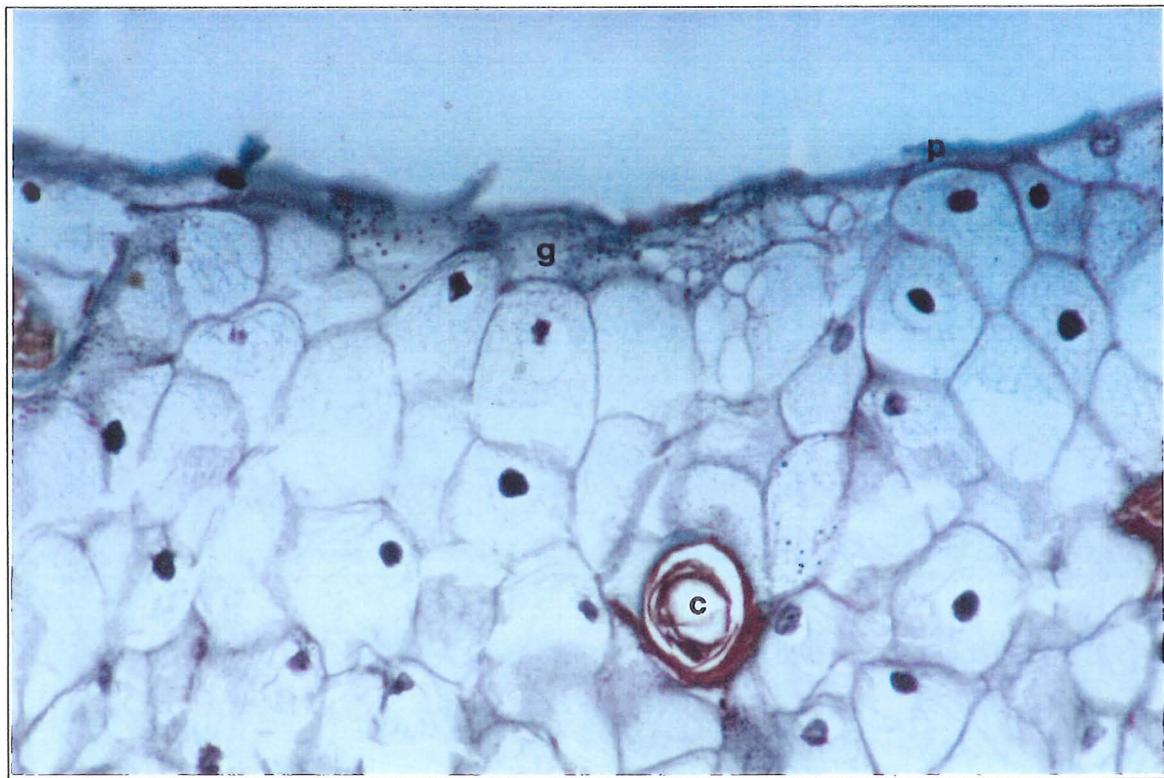


Figure 4.9 154-day-old bovine foetus. Keratinized hair canals (c) are scattered throughout the epidermis. A granular cell layer (g) is present below the periderm (p). Masson's trichrome. X 750.

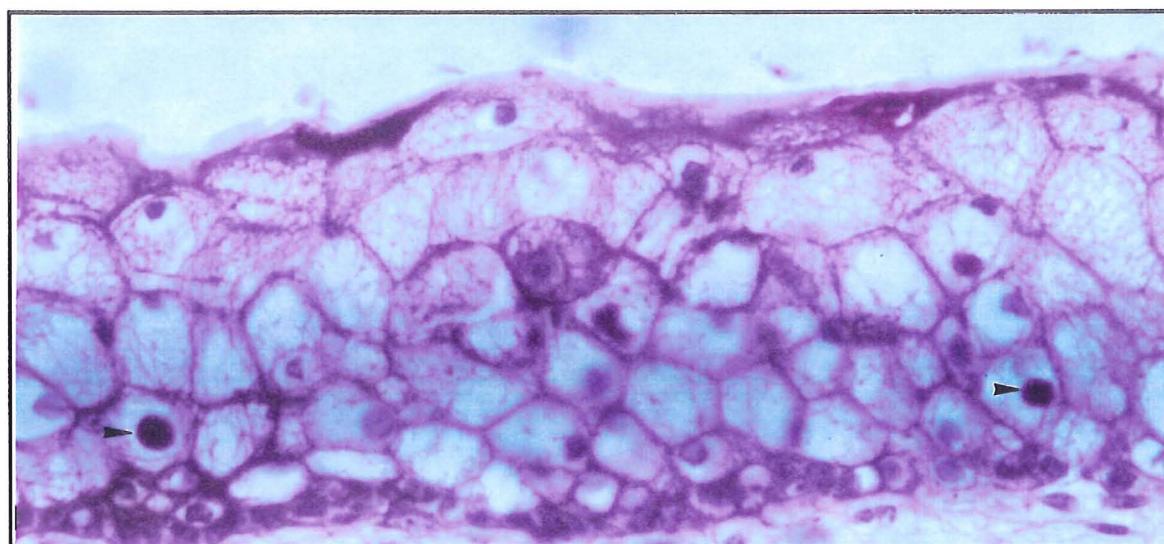


Figure 4.10 154-day-old bovine foetus. Dense masses of glycogen (arrowheads) are seen in some of the stratum intermedium cells near the edge of the horn. Periodic-Acid-Schiff. X 750.

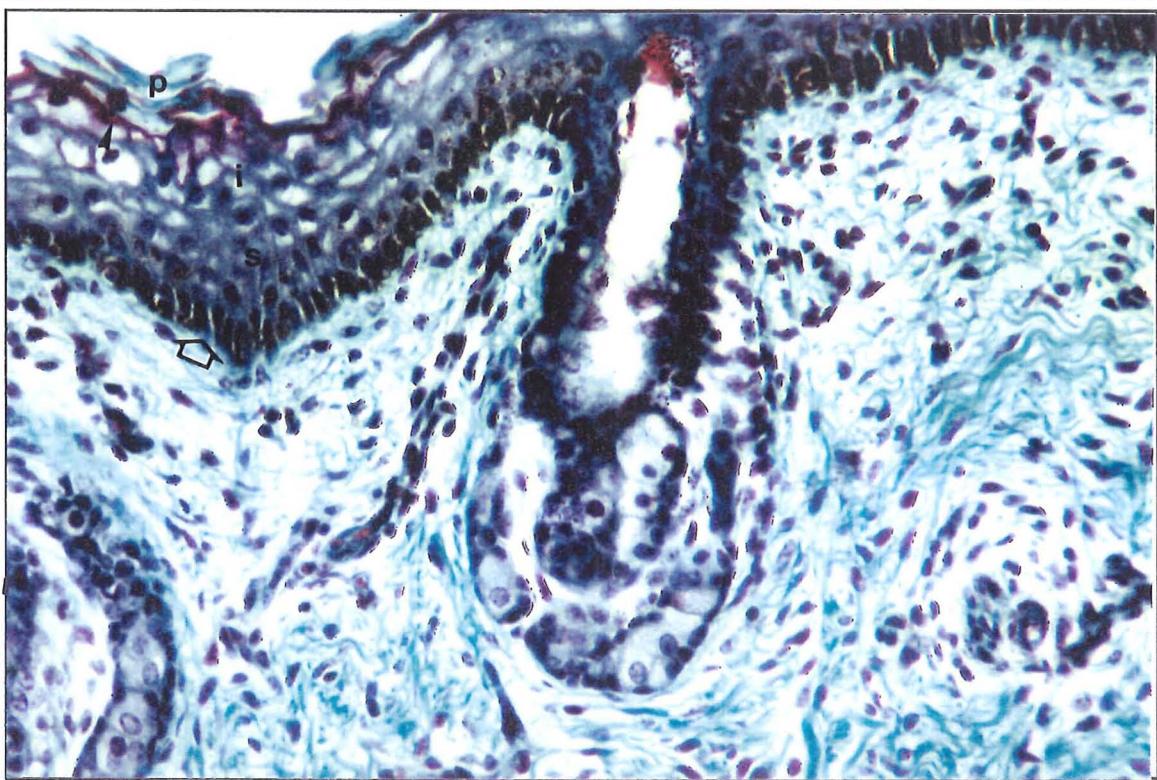


Figure 4.11 169-day-old bovine foetus. Adult-like stratum spinosum cells (s) are seen above the stratum basale (arrow). Immature stratum spinosum cells (i) are seen below the stratum corneum (arrowhead). A few sloughing periderm cells (p) are found above the stratum corneum. Masson's trichrome. X 375.

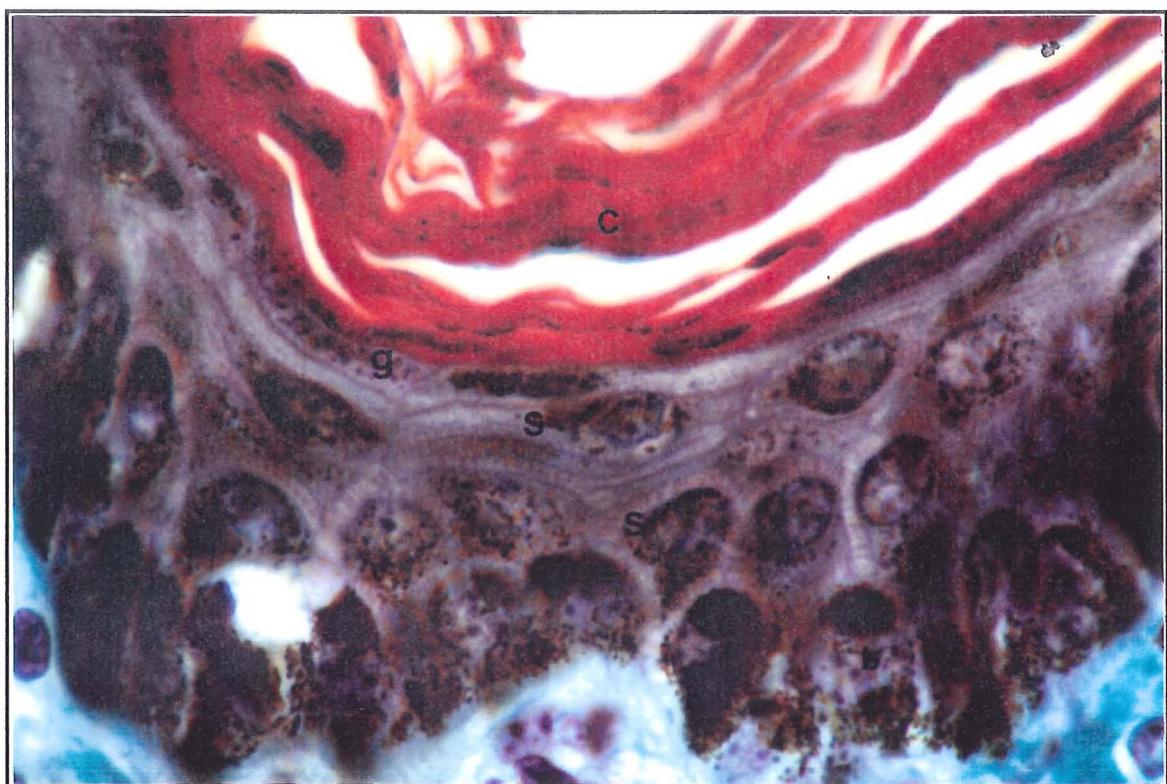


Figure 4.12 204-day-old bovine foetus. Epidermis in the horn bud region. Two layers of spinosal cells (s) are seen above the stratum basale (b). A dequamating stratum corneum (c) is found above the stratum granulosum (g). Masson's trichrome. X 1,875.

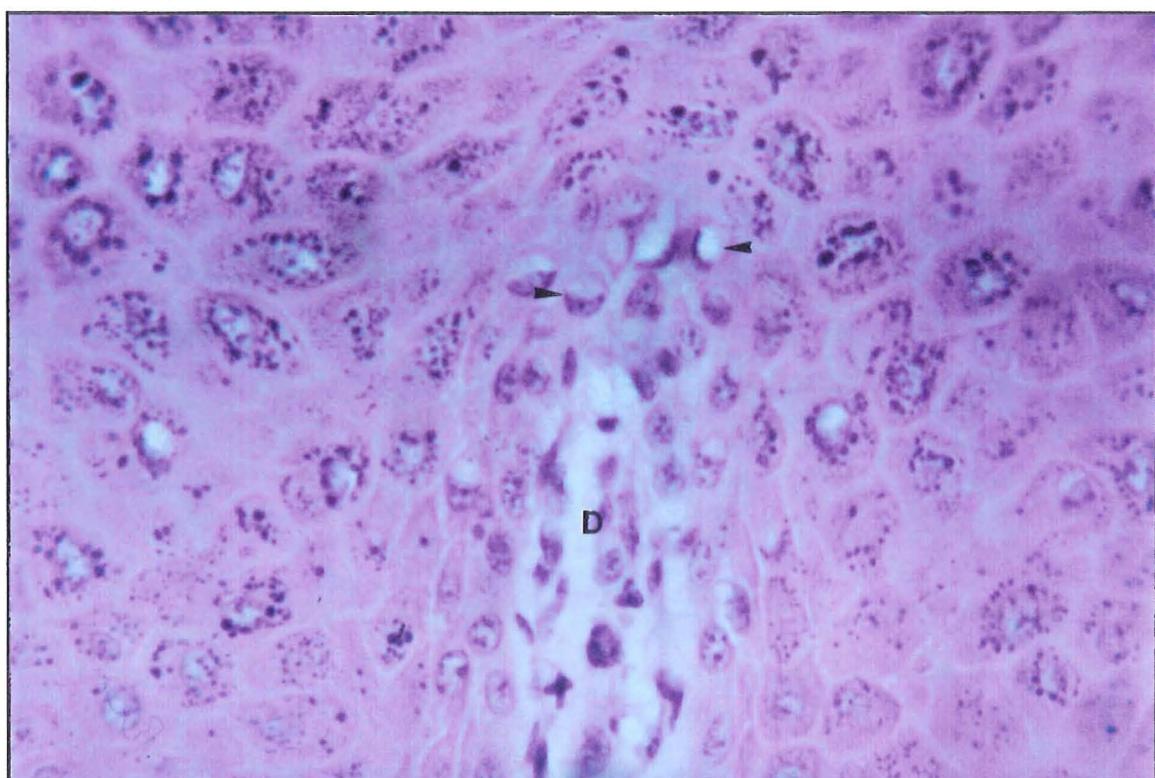


Figure 4.13 2-week-old calf. A developing horn tubule is seen at the tip of a dermal papilla (D). The horn tubule is composed of cells with crescent-shaped nuclei (arrowheads). H&E. X 750.

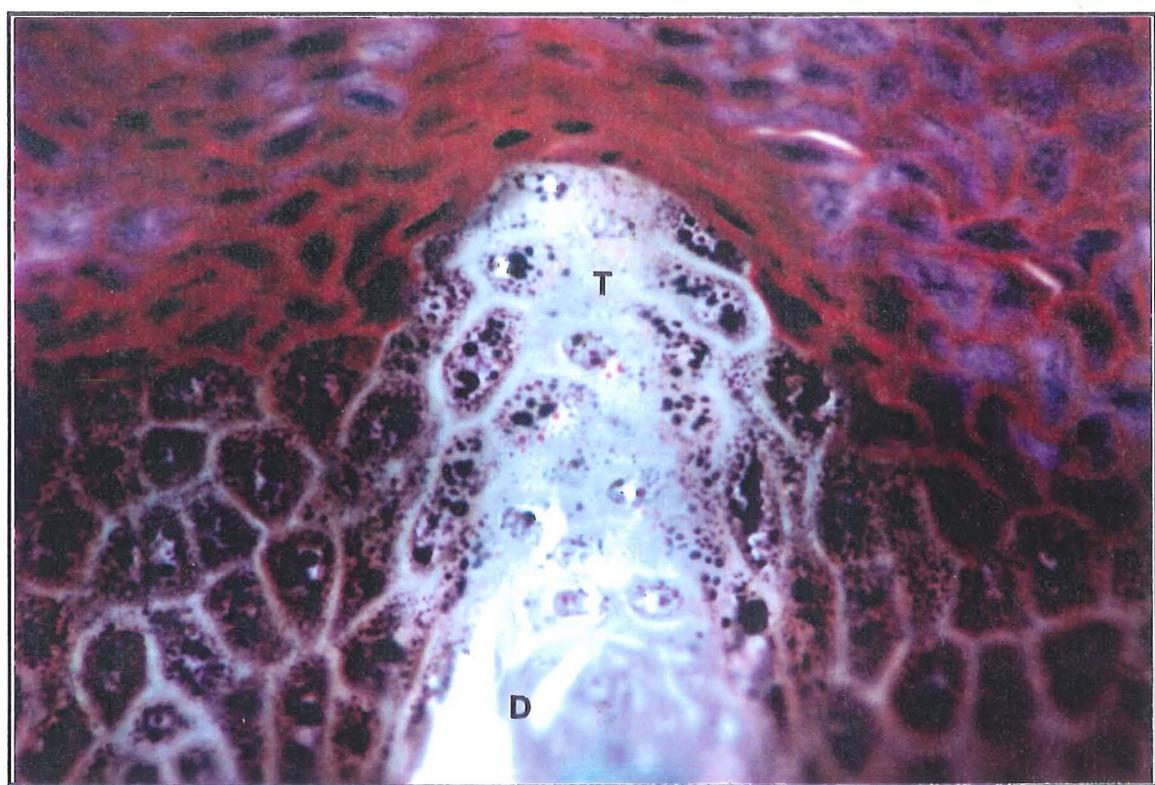


Figure 4.14 10-hour-old kid. Horn tubules (T) develop from cells located over the tips of dermal papillae (D). Masson's trichrome. X 750.

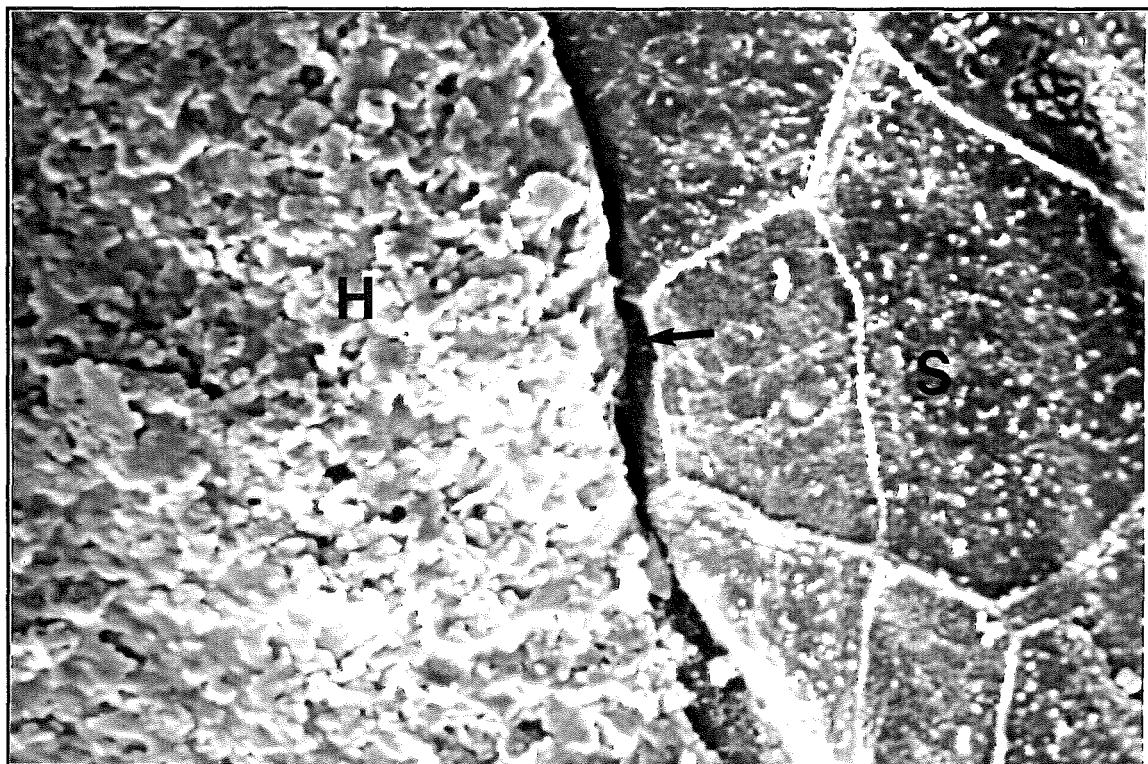


Figure 4.15 71-day-old bovine foetus. Small irregular flakes cover the horn bud area (H), which is separated from the surrounding skin (S) by a deep groove (arrow). Periderm cells with thick borders and numerous microvilli cover the surrounding skin. SEM X 2,800.



Figure 4.16 104-day-old bovine foetus. Protrusions in the groove surrounding the horn bud. A few protrusions (arrows) are seen arising from the centre of the periderm cells. SEM. X 1,440.

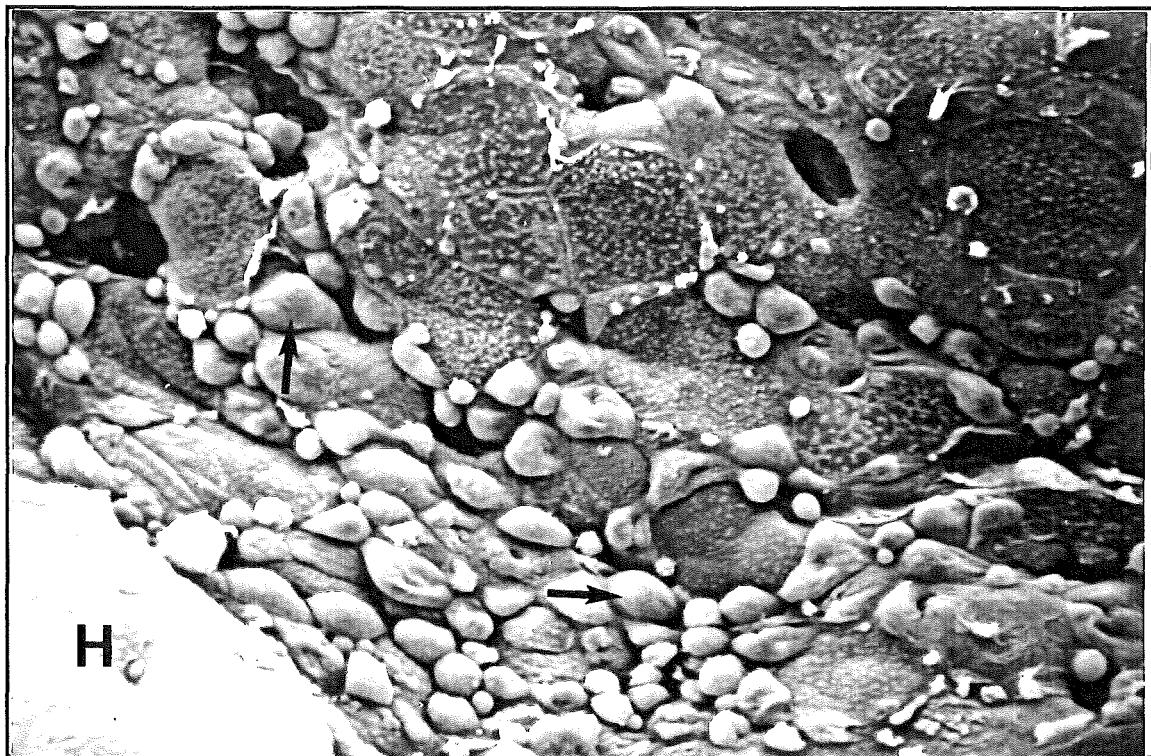


Figure 4.17 112-day-old bovine foetus. The raised horn area (H) is surrounded by a circular groove. Oval protrusions (arrows) are seen arising from the cells lining the groove. Protrusions are more numerous close to the base of the horn bud. SEM. X 720.

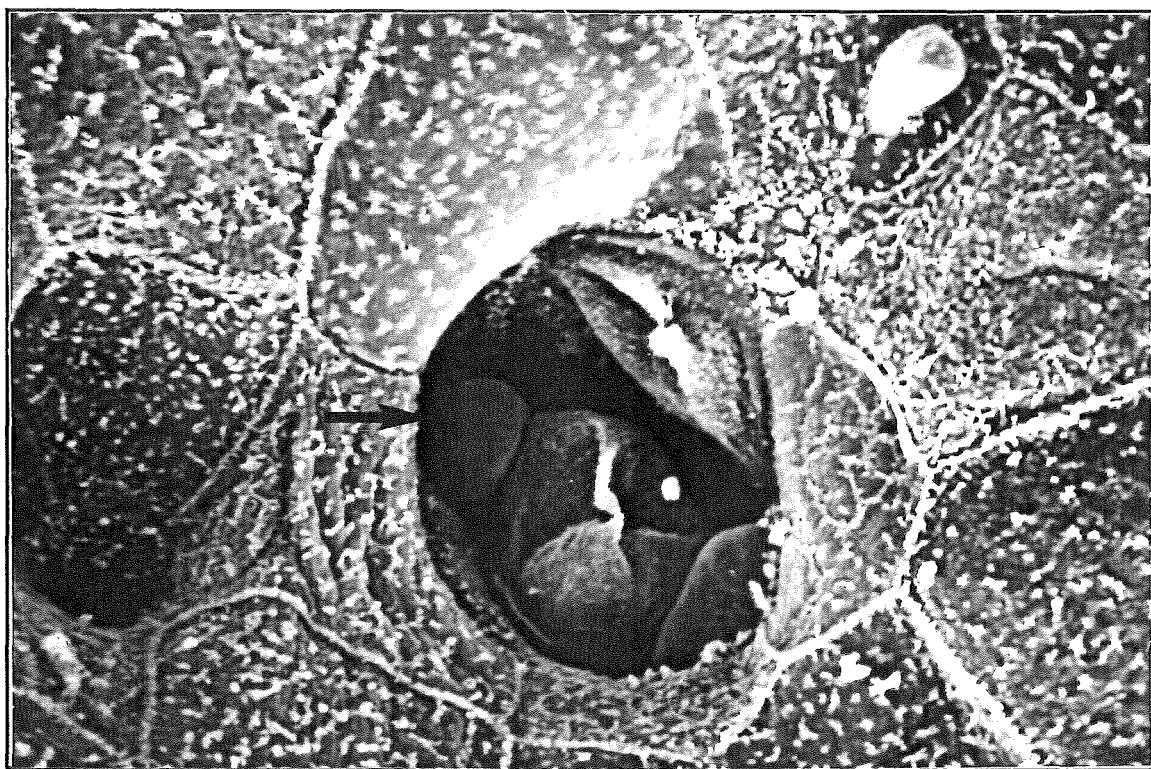


Figure 4.18 125-day-old bovine foetus. Deep cavities (arrow) form in areas where periderm cells have been shed. SEM. X 2,800.

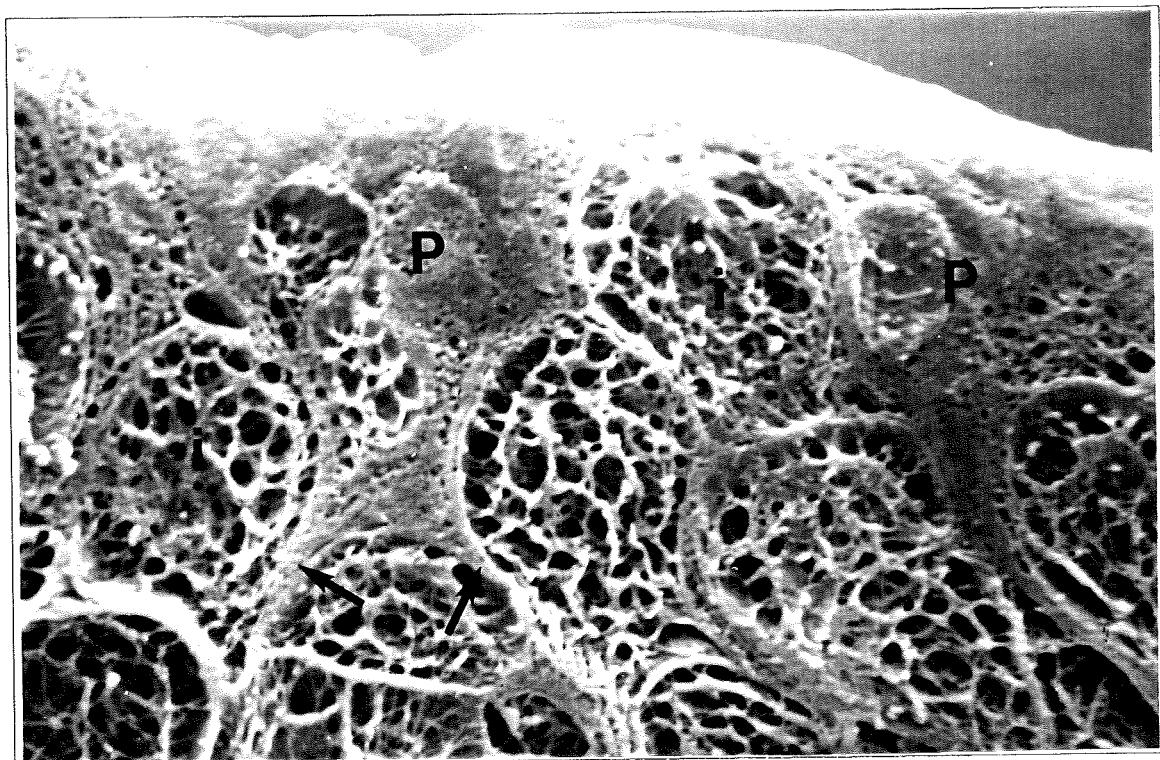


Figure 4.19 125-day-old bovine foetus. Large periderm cells (P) with long tapered cytoplasmic processes (arrows) are seen at the edges of the horn bud. The stratum intermedium (i) cells contain extensive tonofilament networks. SEM. X 2,800.

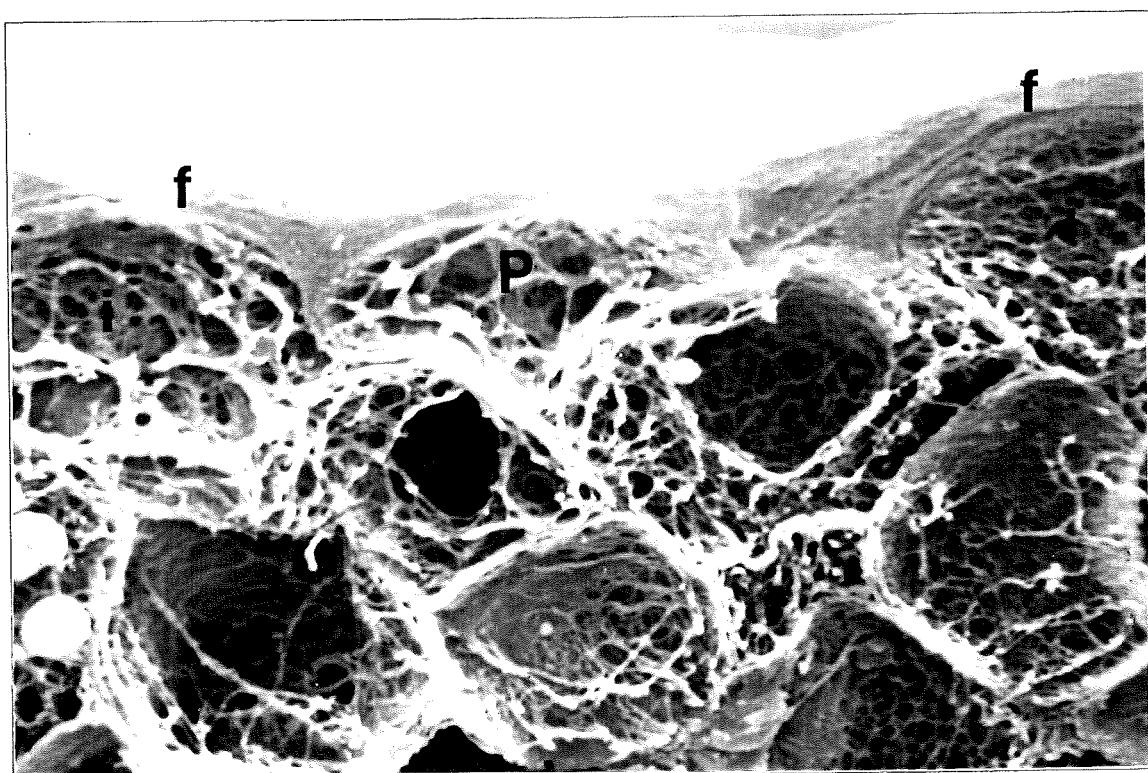


Figure 4.20 125-day-old bovine foetus. A single layer of periderm cells (P) covers the skin area. The periderm cells have broad, flat extensions (f) which cover several intermedium cells (i). The central region of the periderm cells contain tonofilament bundles. SEM. X 2,800.

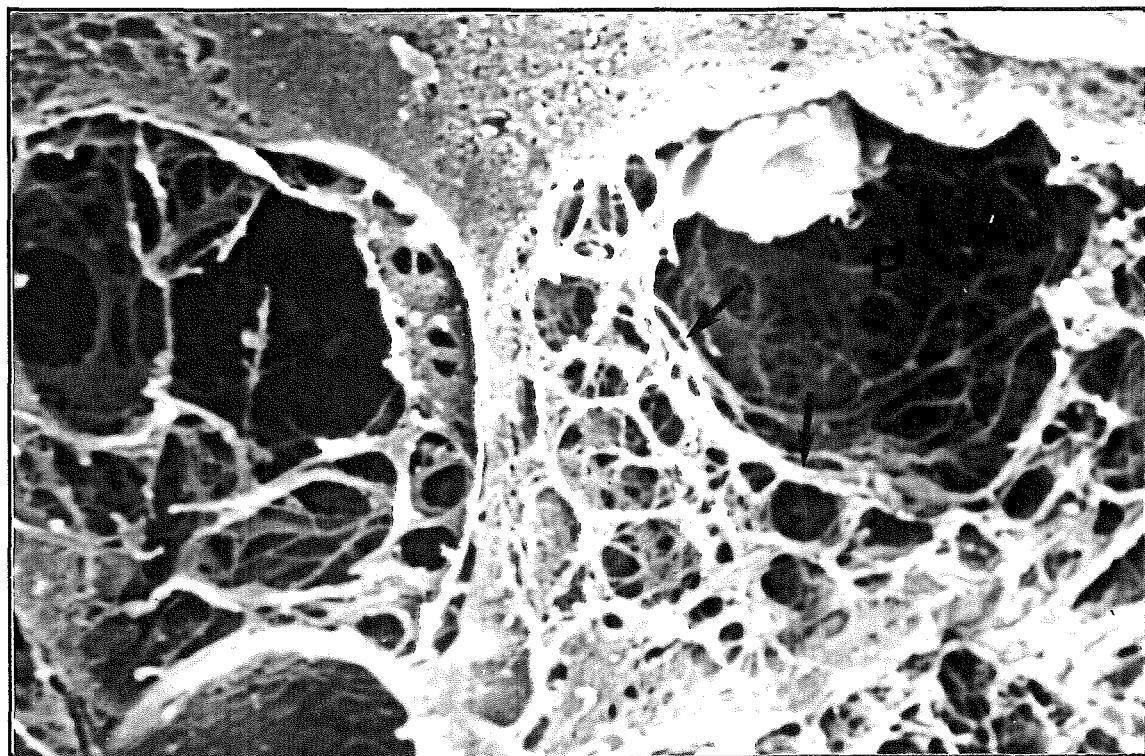


Figure 4.21 125-day-old bovine foetus. A network of cytoplasmic filaments (arrows) occupies most of the intermedium cell. Note the lack of filaments in the perinuclear zone (p). SEM. X 11,250.

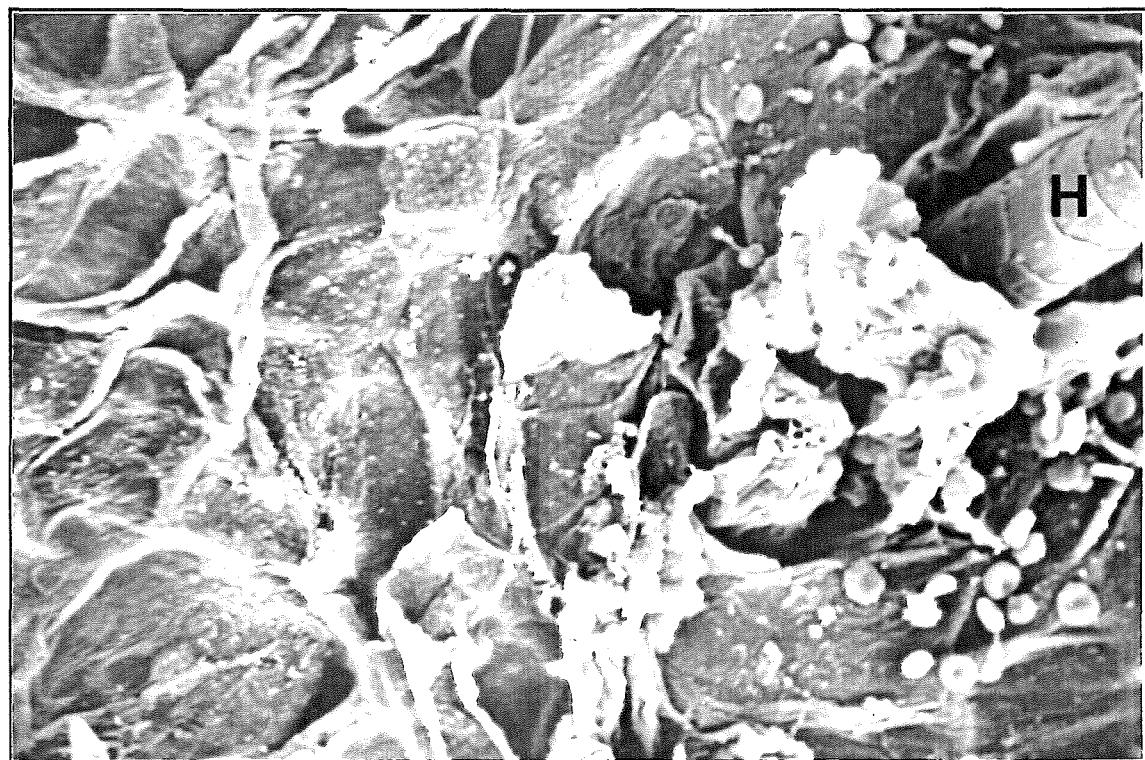


Figure 4.22 169-day-old bovine foetus. A hair (H) penetrates the superficial epidermal layer. SEM. X 1,440.

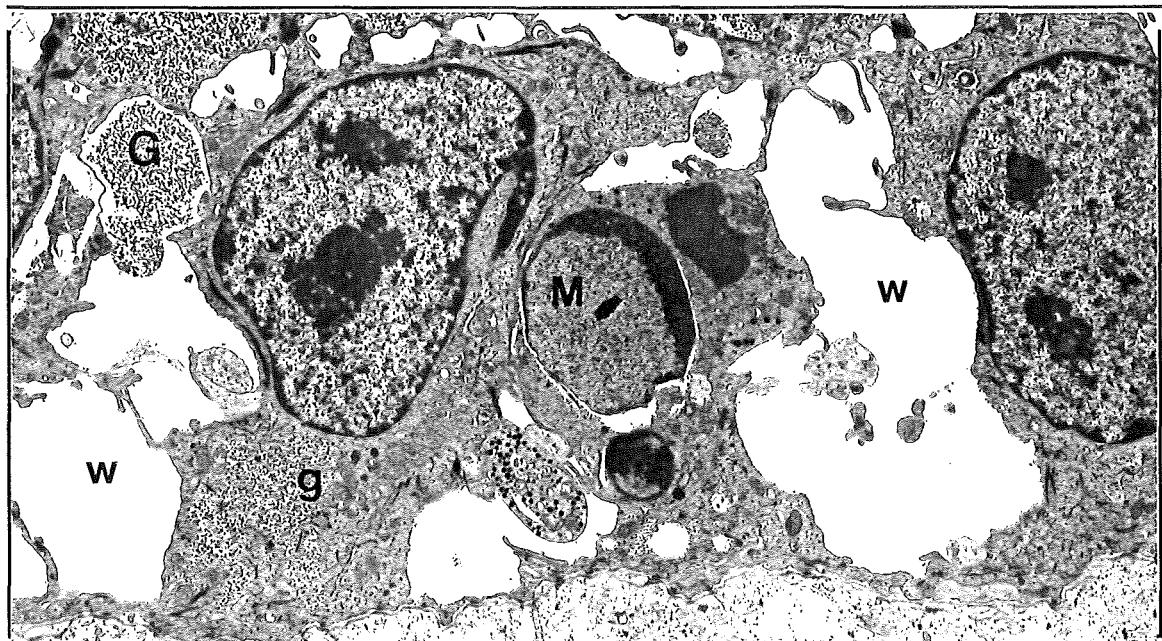


Figure 4.23 71-day-old bovine foetus. Wide spaces (w) are present between the stratum basale cells, one of which has a mitotic nucleus(m). Long cytoplasmic processes extend into the intercellular spaces. Very few desmosomes are seen between adjacent stratum basale cells. Note the presence of intercellular (G) and intracellular (g) glycogen.TEM. X 5,400.

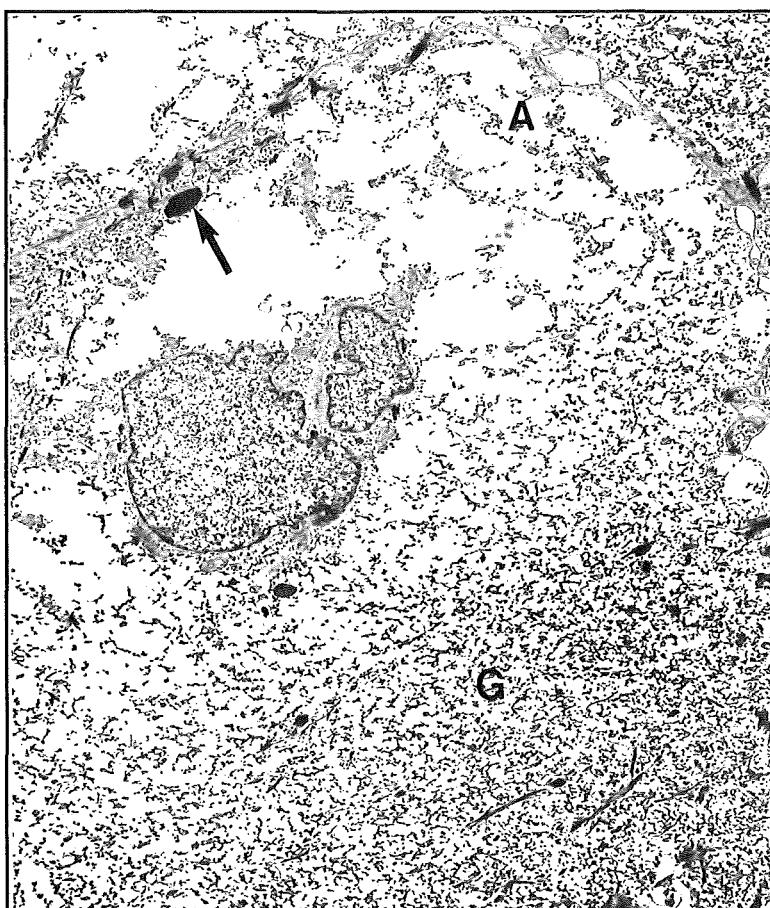


Figure 4.24 71-day-old bovine foetus. Glycogen granules (G) in the stratum intermedium tend to be concentrated in the basal regions of the cells. The glycogen in the apical region (A) of the cell forms anastomosing cords. Note the electron-dense keratin-like granule (arrow) in the intermediate cell. TEM. X 5,400.

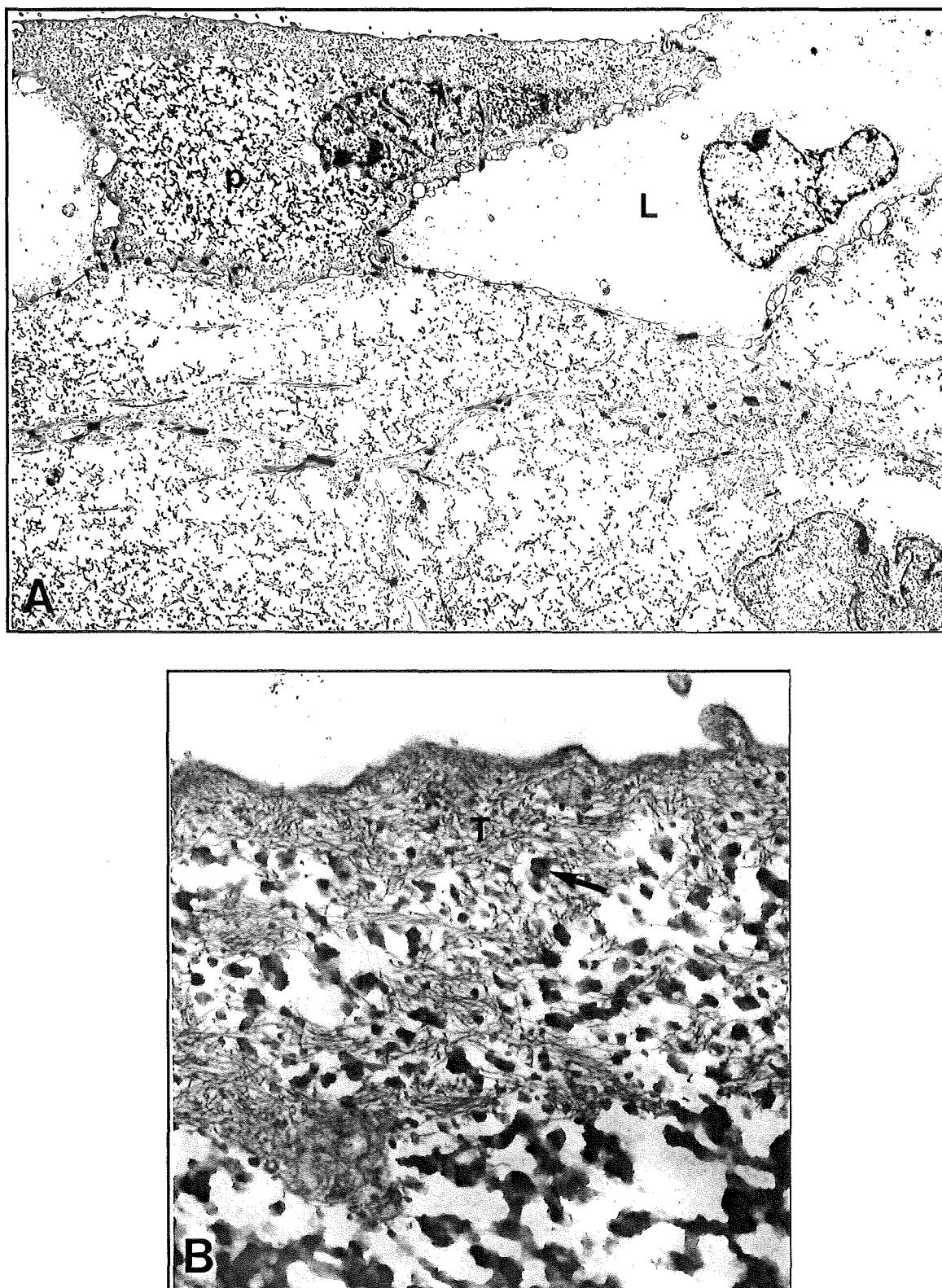


Figure 4.25 71-day-old bovine foetus. A. A glycogen-filled periderm cell (p) with elongated lateral processes interdigitates with an irregular-shaped periderm cell with a more electron-lucent cytoplasm (L). TEM. X 4,000.

B. The periphery of a glycogen-filled periderm cell contains tonofilaments (T) interspersed with glycogen granules (arrow). TEM. X 40,000.

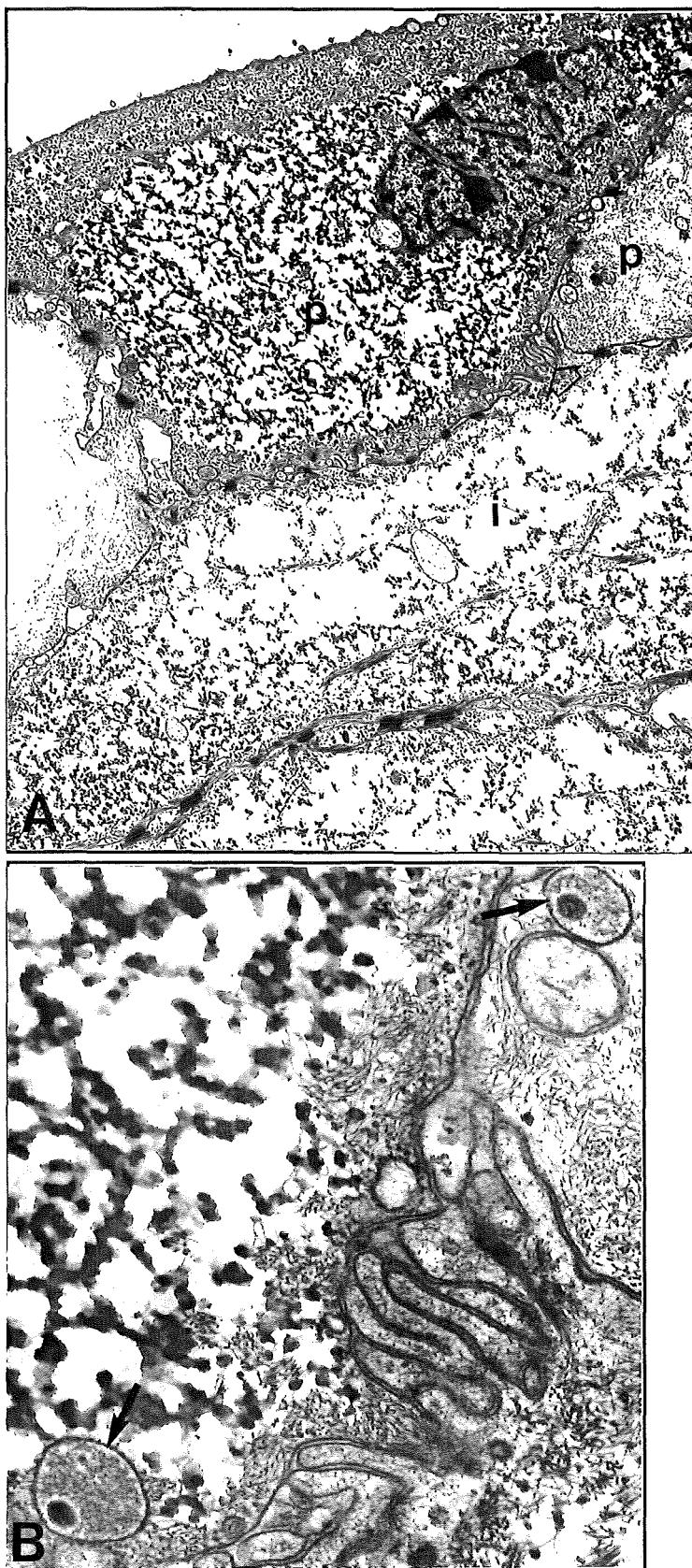


Figure 4.26 71-day-old bovine foetus. **A.** Interdigitating processes (arrow) are seen at the junction of the peridermal (p) and intermediate (i) cell layers. TEM. X5,400.
B. Vesicles (arrows) containing electron-dense bodies are seen close to the periderm cell borders. TEM. X 40,000.

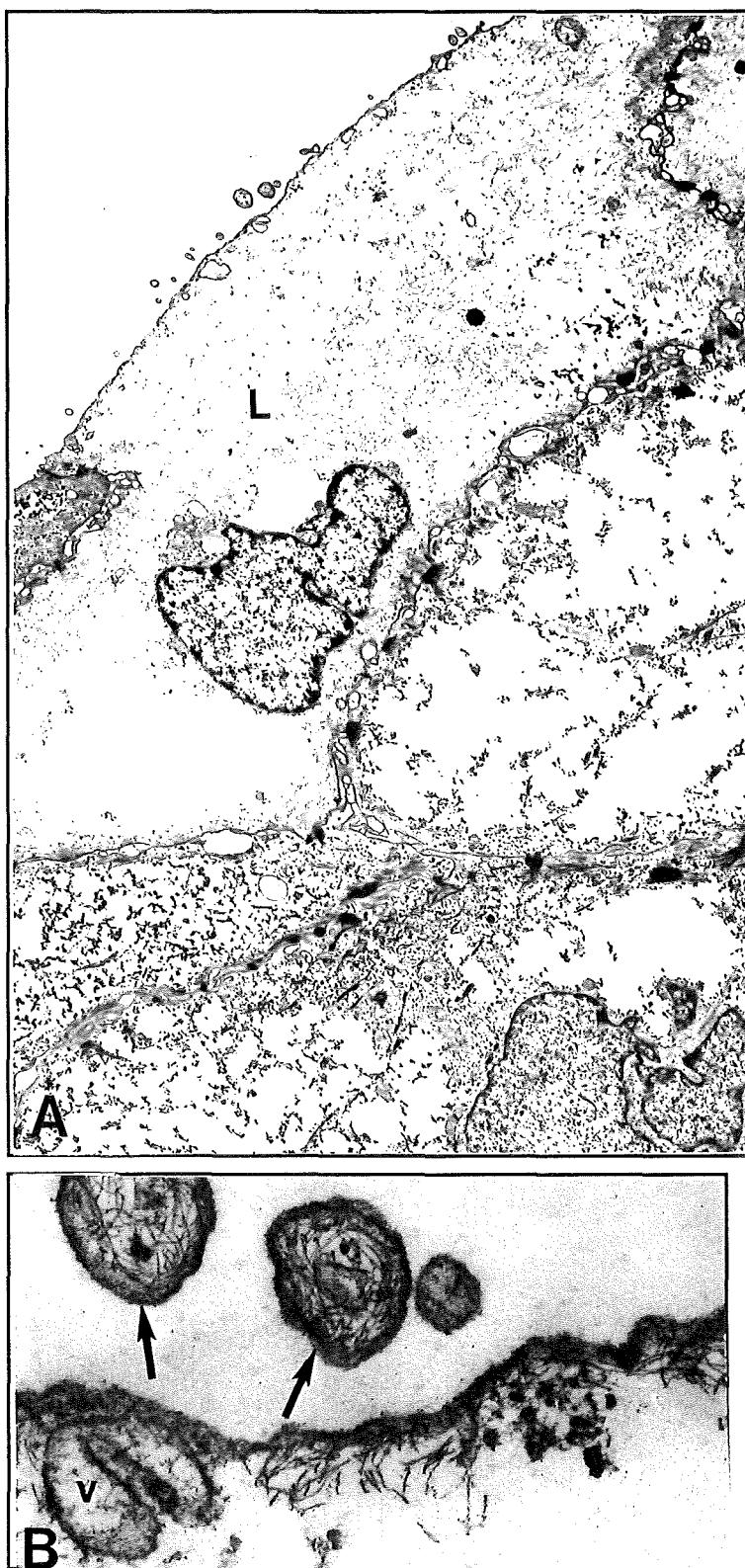


Figure 4.27 71-day-old bovine foetus. A. Several vesicles of various shapes and sizes are present below the apical plasma membrane of the electron-lucent periderm cell (L). TEM. X 5,400.

B. An irregular-shaped vesicle (v) is seen below the surface of the periderm cell shown in A. Transversely cut microvilli (arrows) are also present. The periderm cell contains a scattering of tonofilaments and glycogen granules. TEM X 40,000.

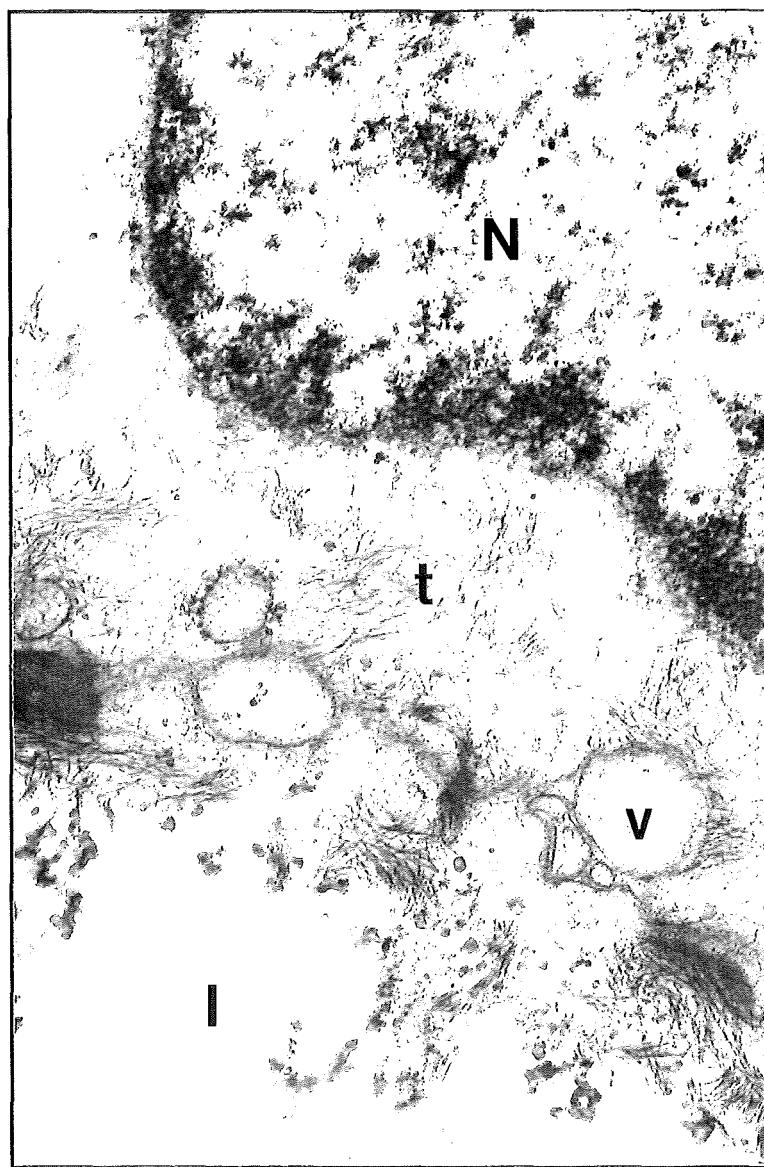


Figure 4.28 71-day-old bovine foetus. Tonofilaments (t) and vesicles (v) are observed between the nucleus (N) of an electron-lucent periderm cell and the apical plasma membrane of an intermediate cell (l). TEM. X 40,000.



Figure 4.29 71-day-old bovine foetus. Two mesenchymal cells (M) in the papillary region of the skin are linked by desmosome-like connections (arrows). TEM. X 26,000.

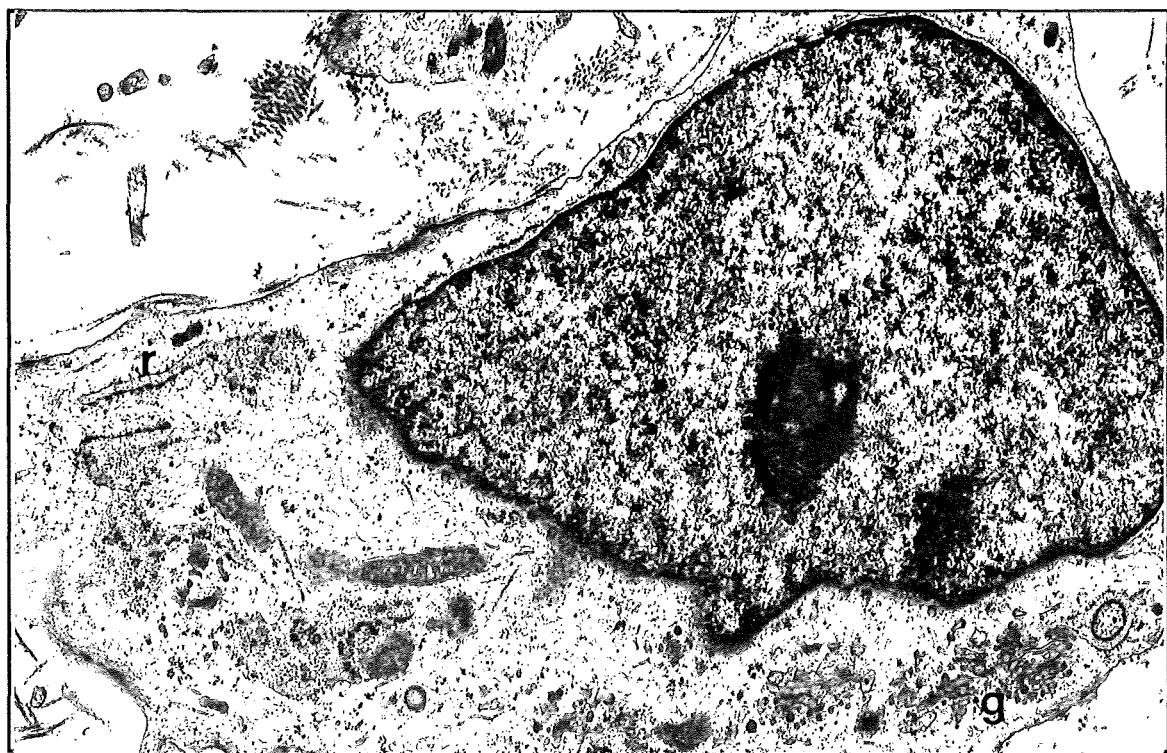


Figure 4.30 71-day-old bovine foetus. Mesenchymal cell containing a well-developed Golgi complex (g), and a small amount of rough endoplasmic reticulum (r). Cytofilaments and polyribosomes are scattered throughout the cell. TEM. X 16,000.

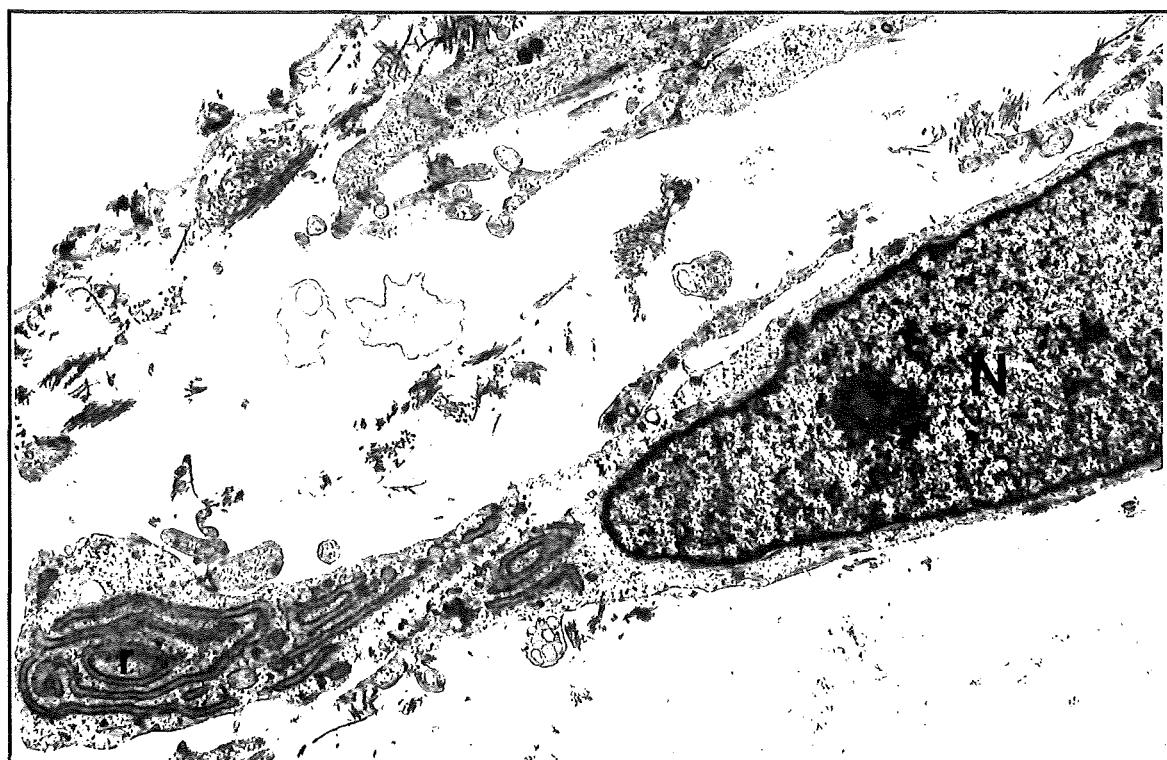


Figure 4.31 71-day-old bovine foetus. The fibroblast is characterized by the presence of well-developed rough endoplasmic reticulum (r) and an elongated nucleus (N). TEM. X 10,000.

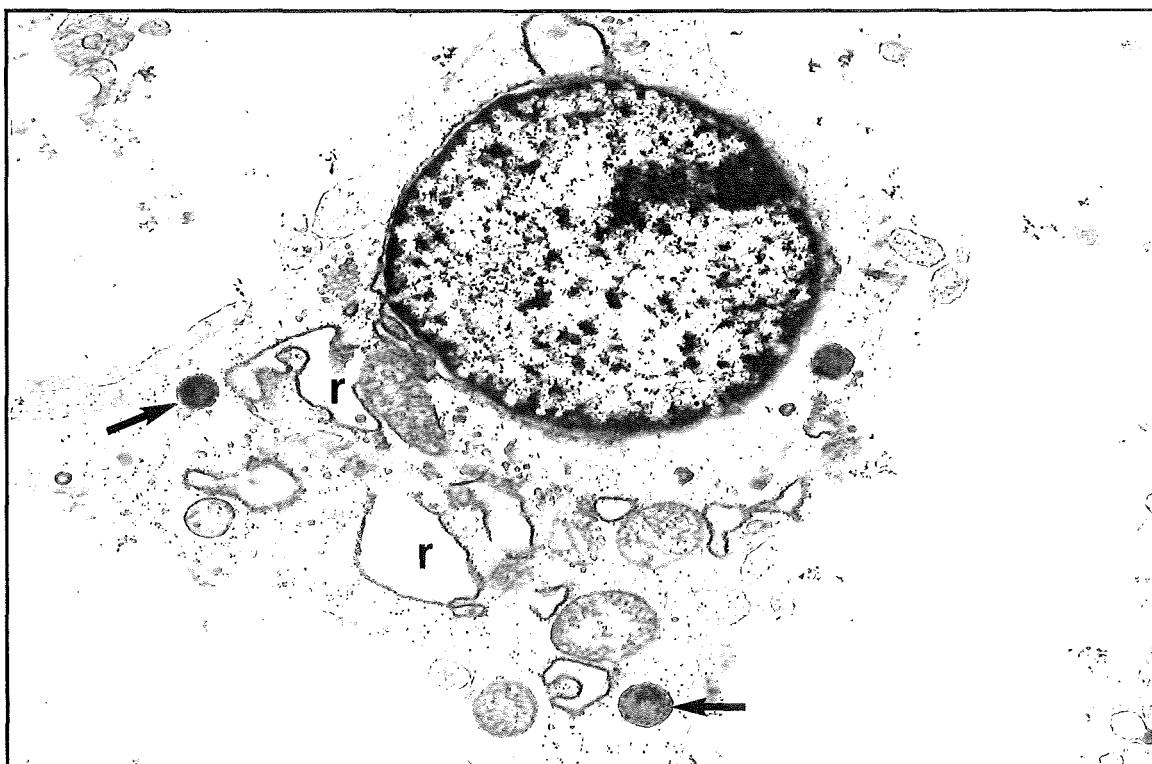


Figure 4.32 71-day-old bovine foetus. Occasional mast cell precursors are found in the dermis. These cells contain dilated rough endoplasmic reticulum (r), numerous small vesicles and a few round electron-dense bodies (arrows). TEM. X 13,400.

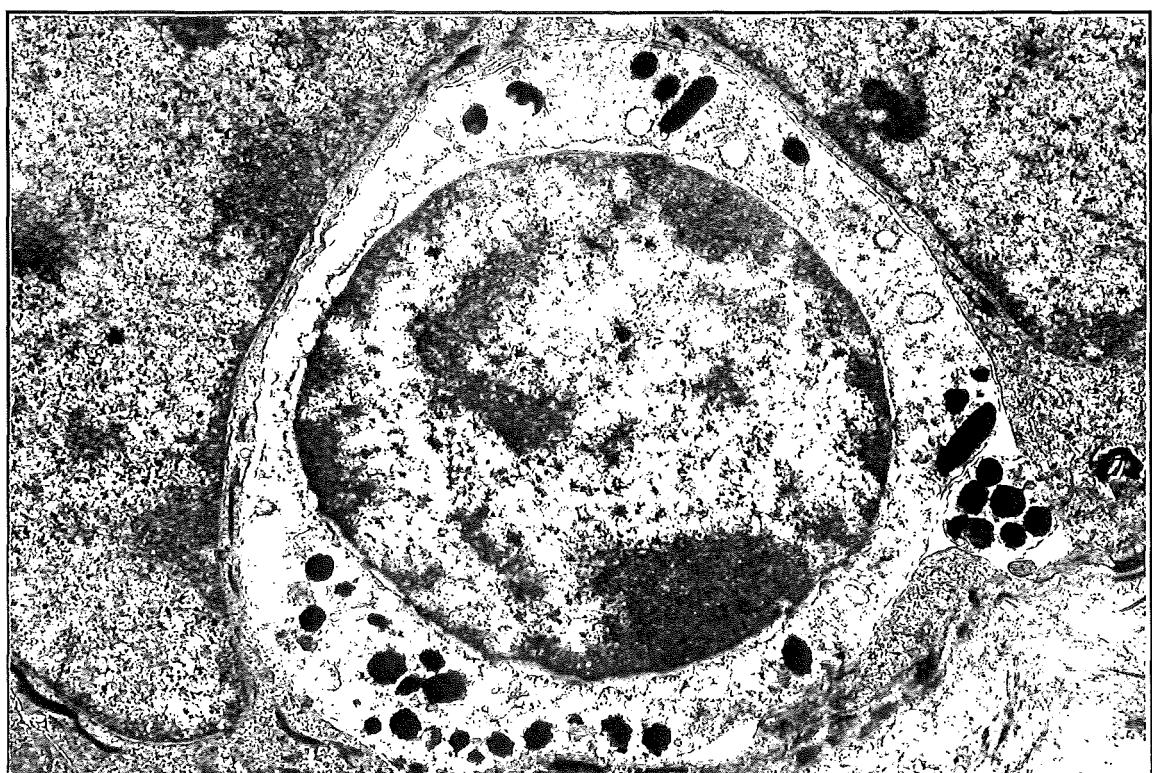


Figure 4.33 104-day-old bovine foetus. A melanocyte with a round nucleus and electron-lucent cytoplasm containing melanosomes is seen within the stratum basale. TEM. X 16,000.



Figure 4.34 104-day-old bovine foetus. A dendritic cell (D) is observed between stratum intermedium cells (I). The cell is presumed to be a Langerhans cell due to the presence of several small electron-dense granules (arrows), as well as the presence of cytoplasmic dendrites. The presumptive Langerhans cell appears to have engulfed part of an adjacent intermedium cell (G). Note the lack of tonofilaments in the cell and the absence of desmosomes linking it to adjacent cells. TEM. X16,400.



Figure 4.35 104-day-old bovine foetus. Microvilli, some of which are branched (arrow), are seen on the surface of the periderm. The microvilli contain filamentous bundles. The remainder of the periderm cell is occupied by tonofilaments and a few glycogen granules. TEM. X 20,000.

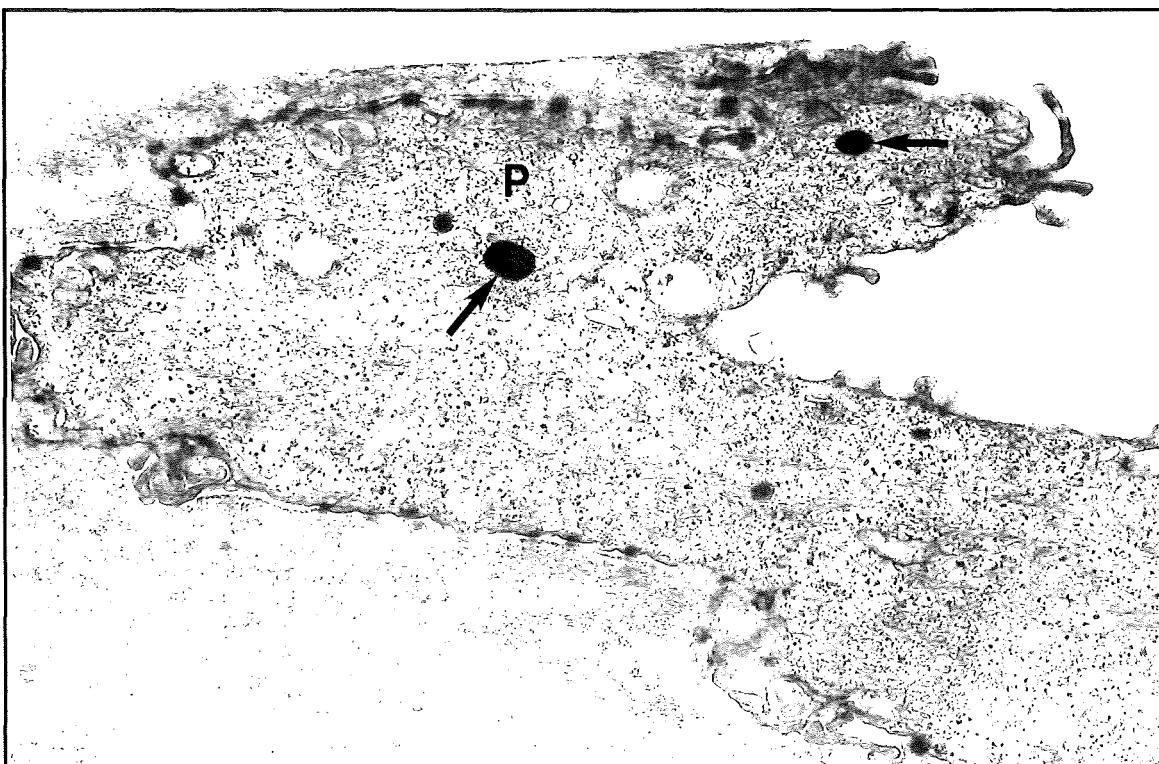


Figure 4.36 105-day-old bovine foetus. An elongated protrusion (P) extends from the periderm surface. Note that the protrusion involves two adjacent periderm cells. Electron-dense bodies (arrows) are seen within the protrusion. TEM. X 16,000.

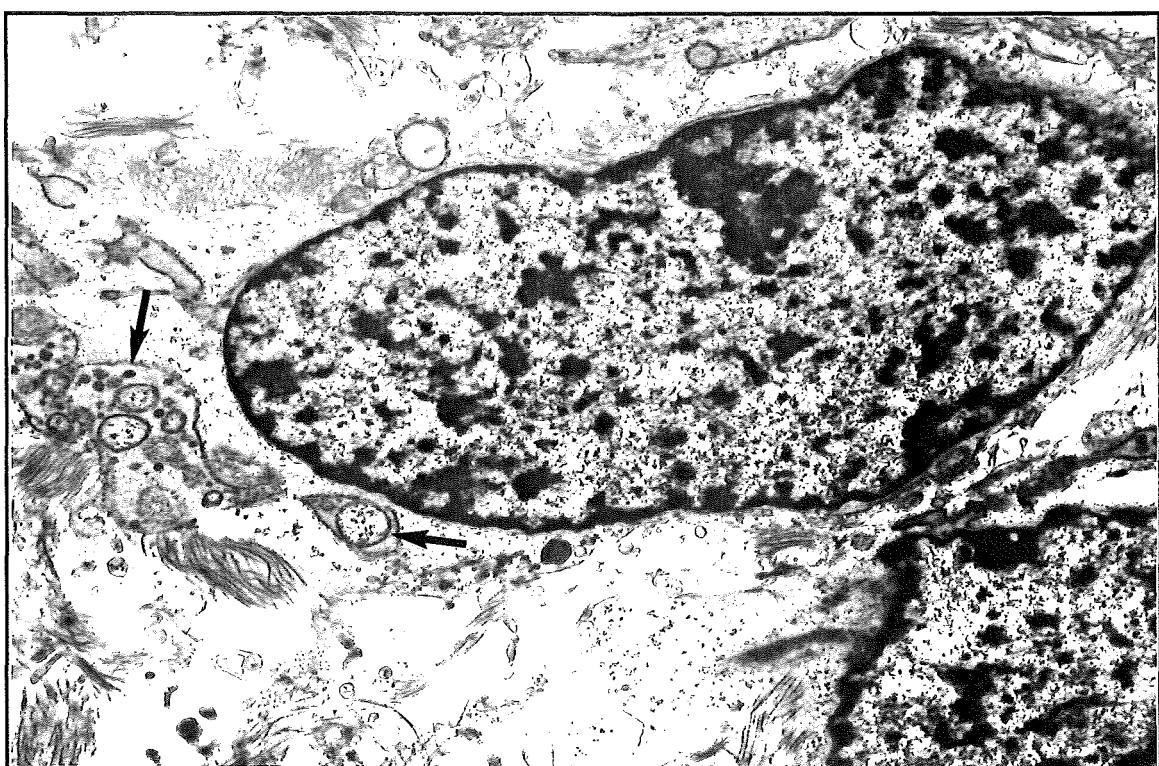


Figure 4.37 105-day-old bovine foetus. The spindle-shaped fibroblast has an elongated nucleus surrounded by cytoplasm which is filled with rough endoplasmic reticulum and cytofilaments. The rough endoplasmic reticulum encloses several vesicles (arrows) of various sizes. TEM. X 10,000.

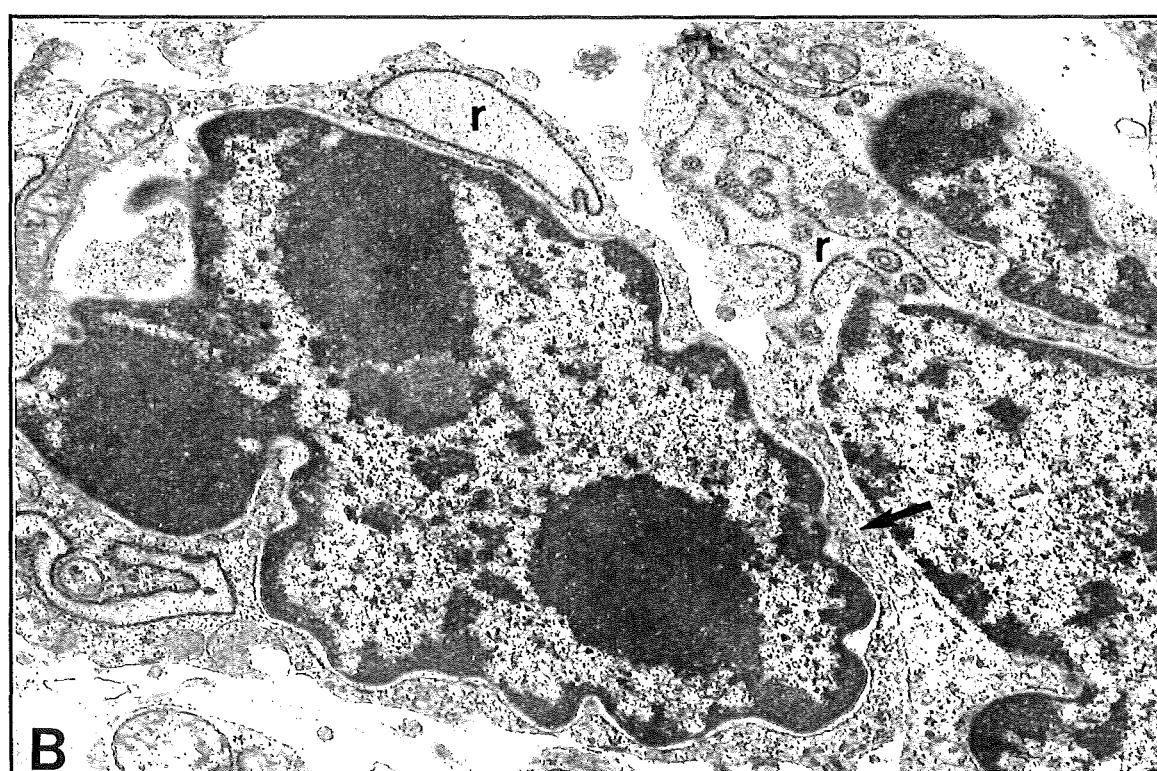
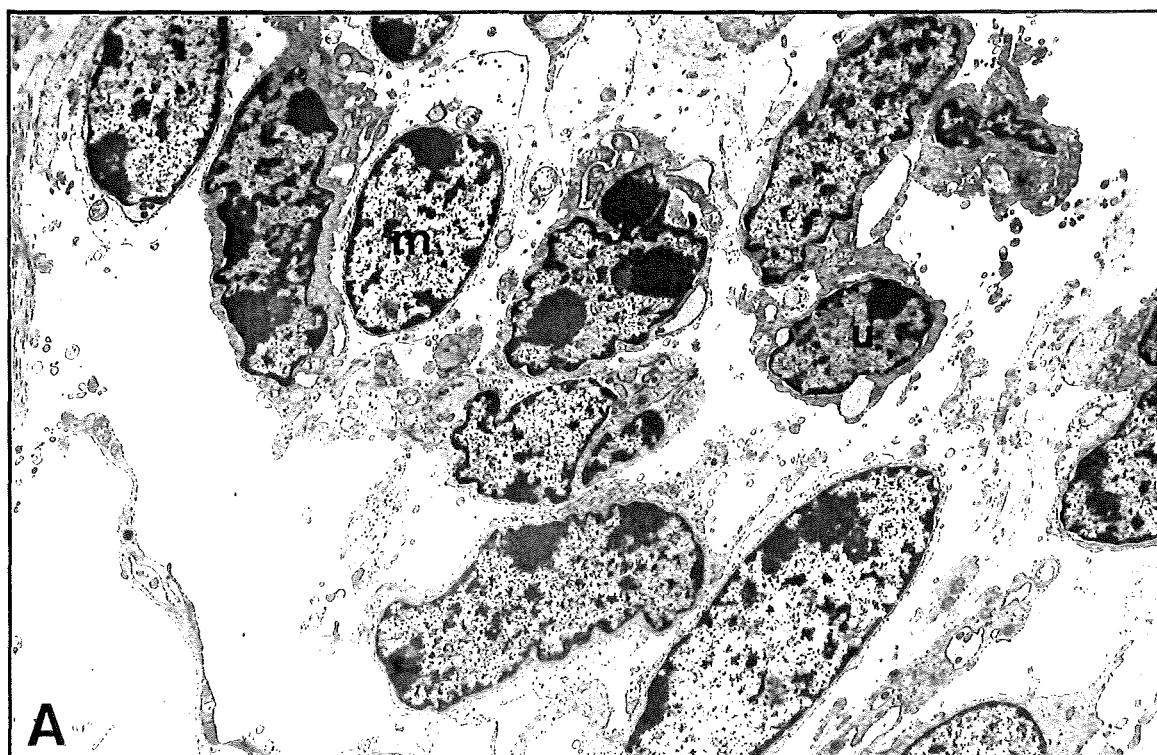


Figure 4.38 105-day-old bovine foetus. A. A collection of undifferentiated (u) and maturing (m) mesenchymal cells in the papillary region of skin. TEM X5,400.

B. Two mesenchymal cells are in close contact over a long section of plasma membrane (arrow). Several dilated rough endoplasmic reticulum profiles (r) containing small vesicles are seen. TEM. X 20,000.

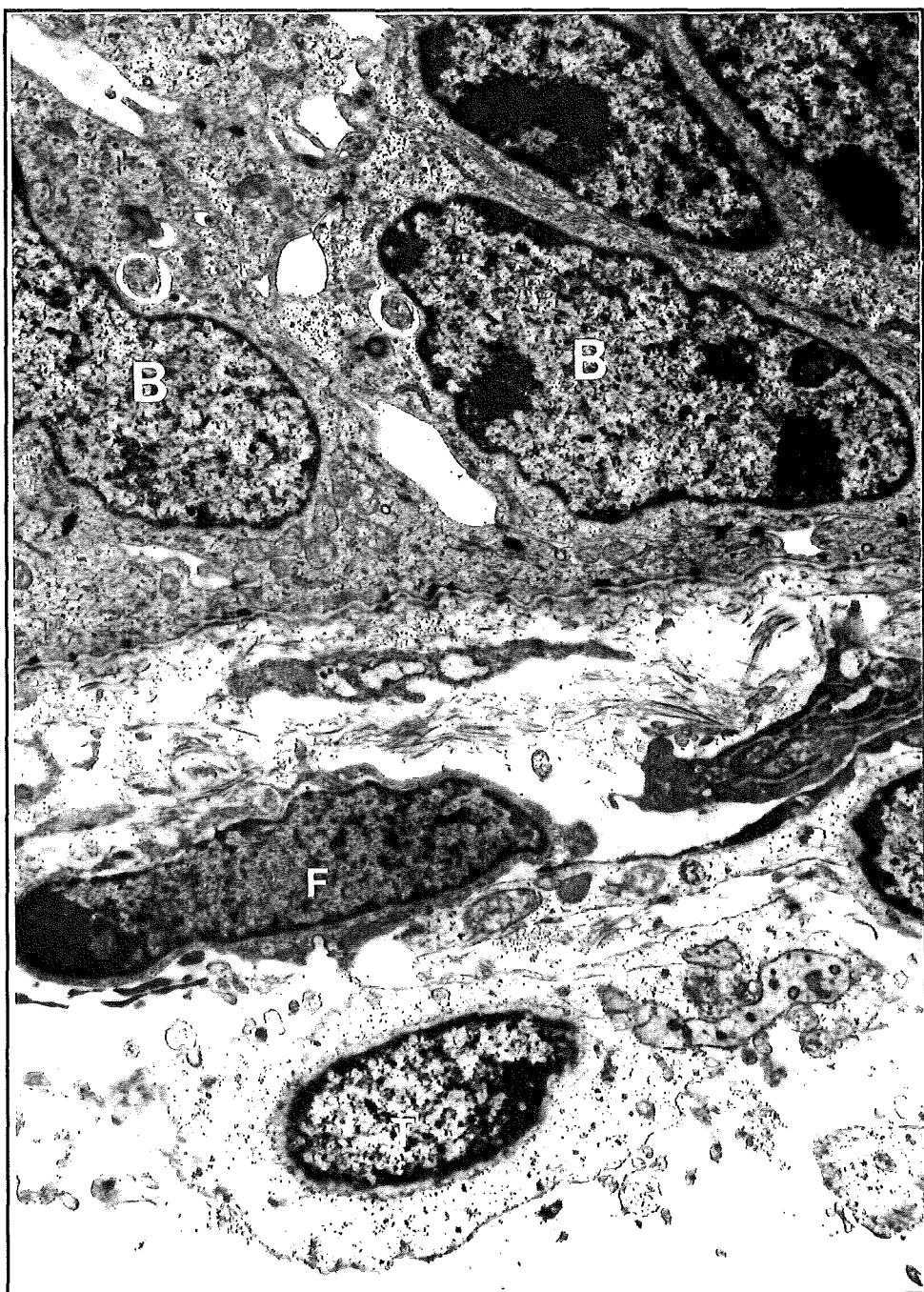


Figure 4.39 117-day-old bovine foetus. Mitochondria and ribosomes are seen within the stratum basale cells (B). A fibroblast (f) with electron-dense cytoplasm is present in the subepidermal region. In the vicinity of the fibroblast is a transitional cell (T) which has characteristics of both fibroblasts and mesenchymal cells.
TEM. X 12,000.

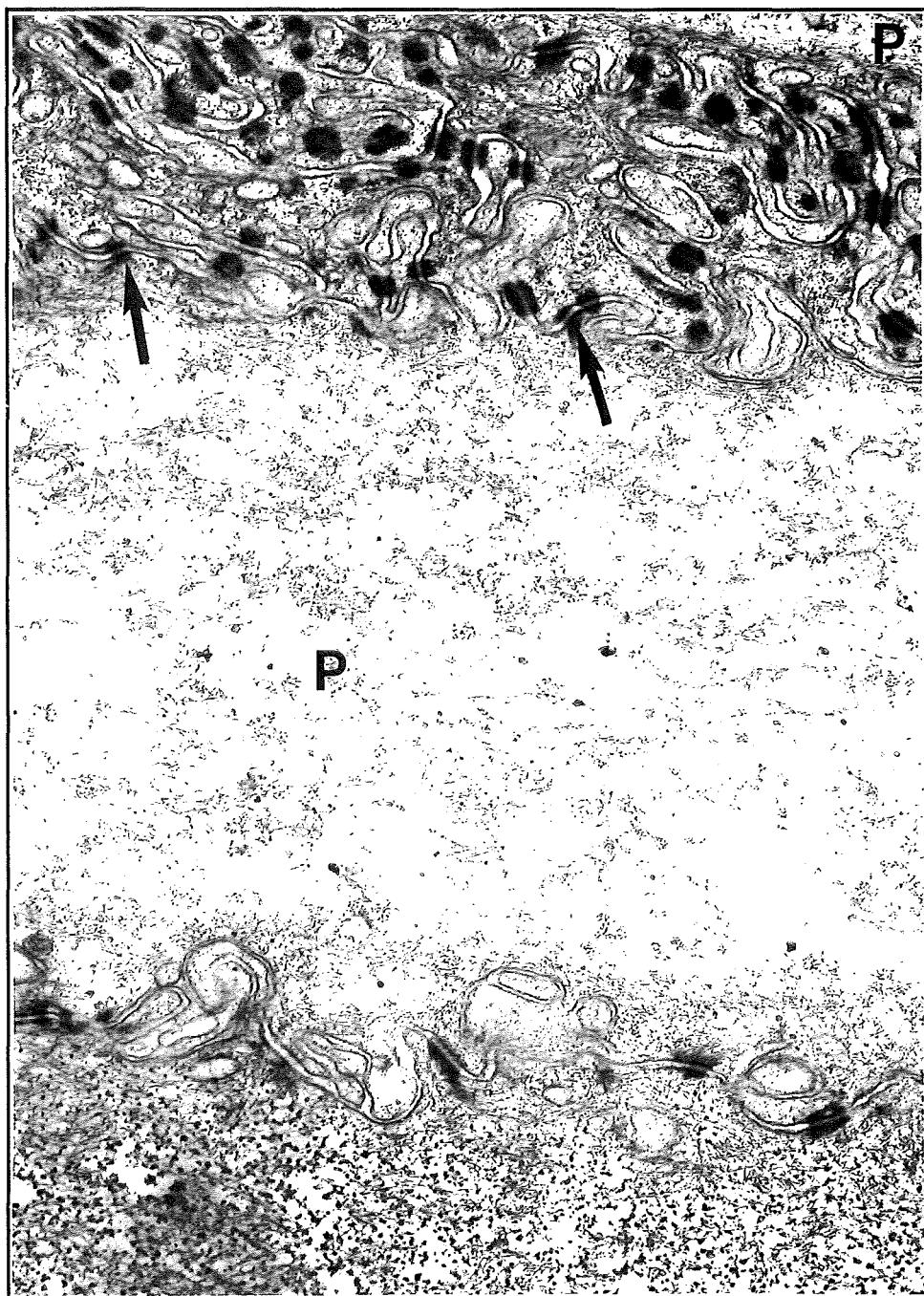


Figure 4.40 117-day-old bovine foetus. Interdigitating processes of the two periderm layers (P) are linked by numerous short desmosomes (arrows). TEM. X 30,000.

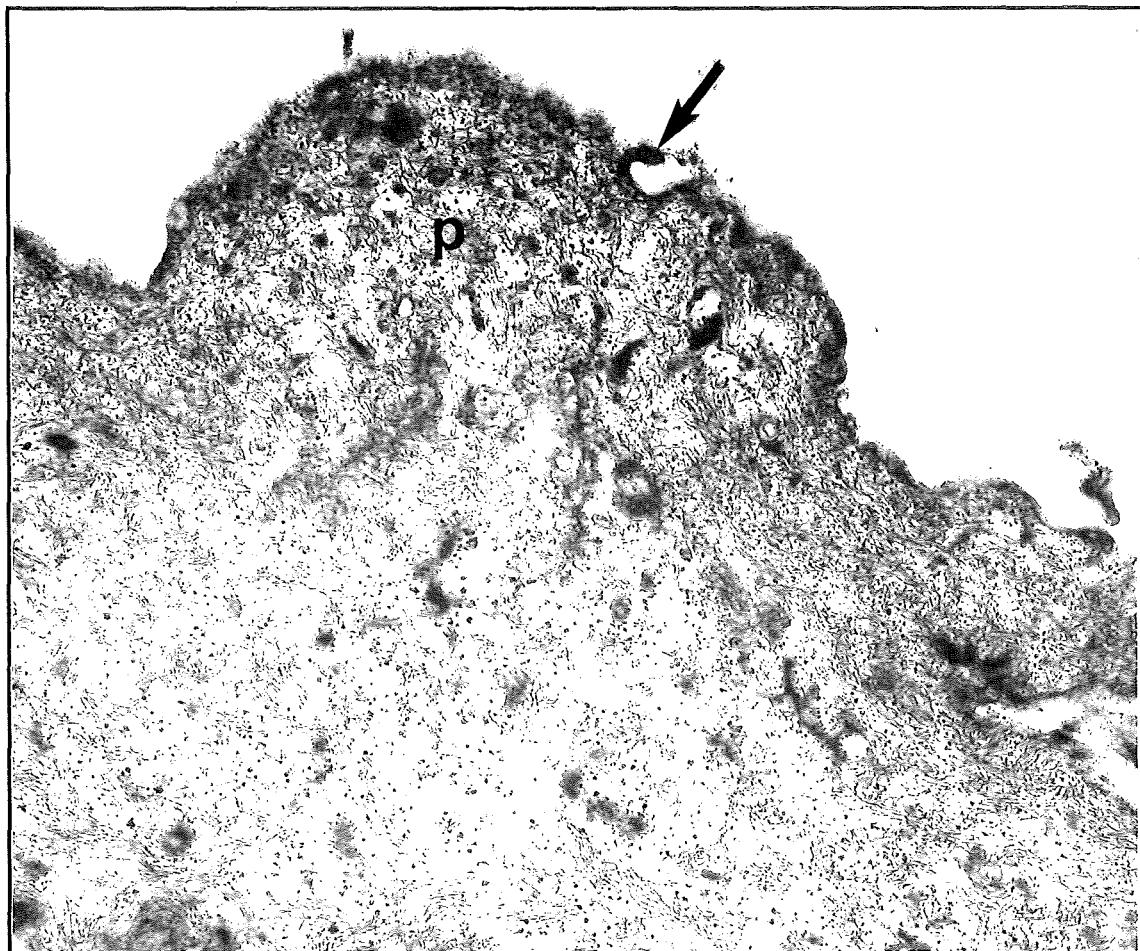


Figure 4.41 117-day-old bovine foetus. In some areas protrusions (p) arise from single periderm cells. The protrusion shown lacks a distinct plasma membrane although a few microvilli are still present (arrow). TEM. X 26,000.



Figure 4.42 122-day-old bovine foetus. The intermedium cell contains glycogen most of which has a reticular arrangement (R). Glycogen granules and tonofilaments are absent from the perinuclear area (P). TEM. X 8,000.

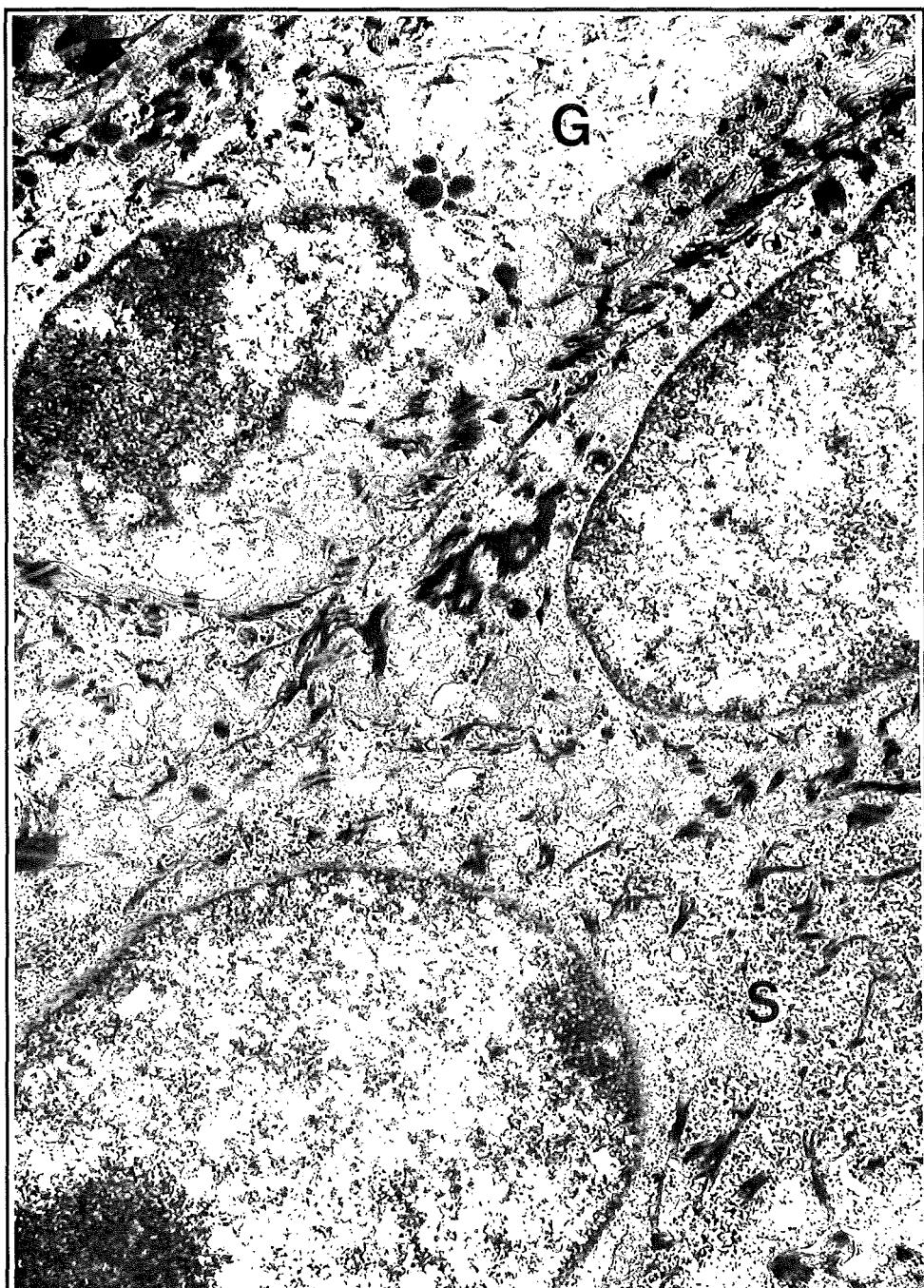


Figure 4.43 203 -day-old bovine foetus. Underlying the stratum corneum (arrow) is a stratum granulosum cell (G) containing a degenerating nucleus and several round electron-dense bodies. Note the stratum spinosum cell (S) containing glycogen granules interspersed with tonofilament bundles. TEM. X 42,000.

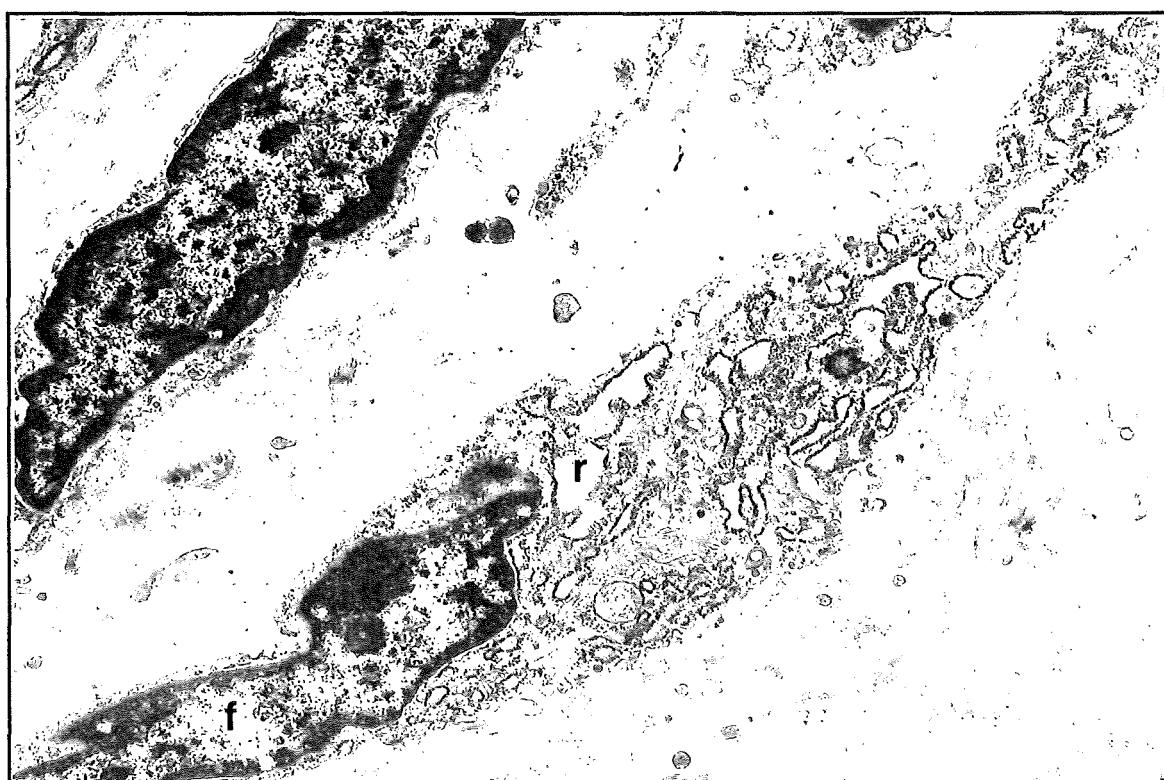


Figure 4.44 198-day-old bovine foetus. Mature fibroblast (f) containing an elongated nucleus surrounded by abundant cytoplasm in which a large amount of rough endoplasmic reticulum (r) is seen. TEM X 13,400.

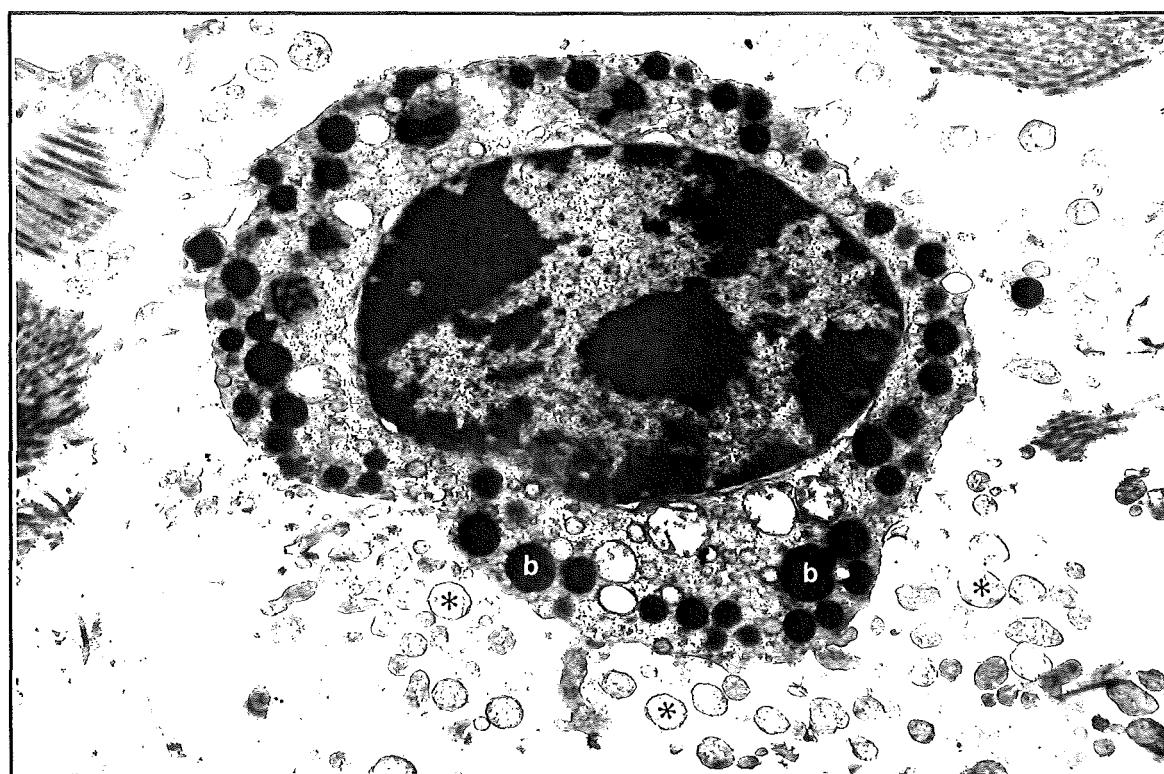


Figure 4.45 198-day-old bovine foetus. The presence of numerous cytoplasmic processes (*) and electron-dense bodies (b) suggests that this cell is a mast cell. TEM. X 13,400.

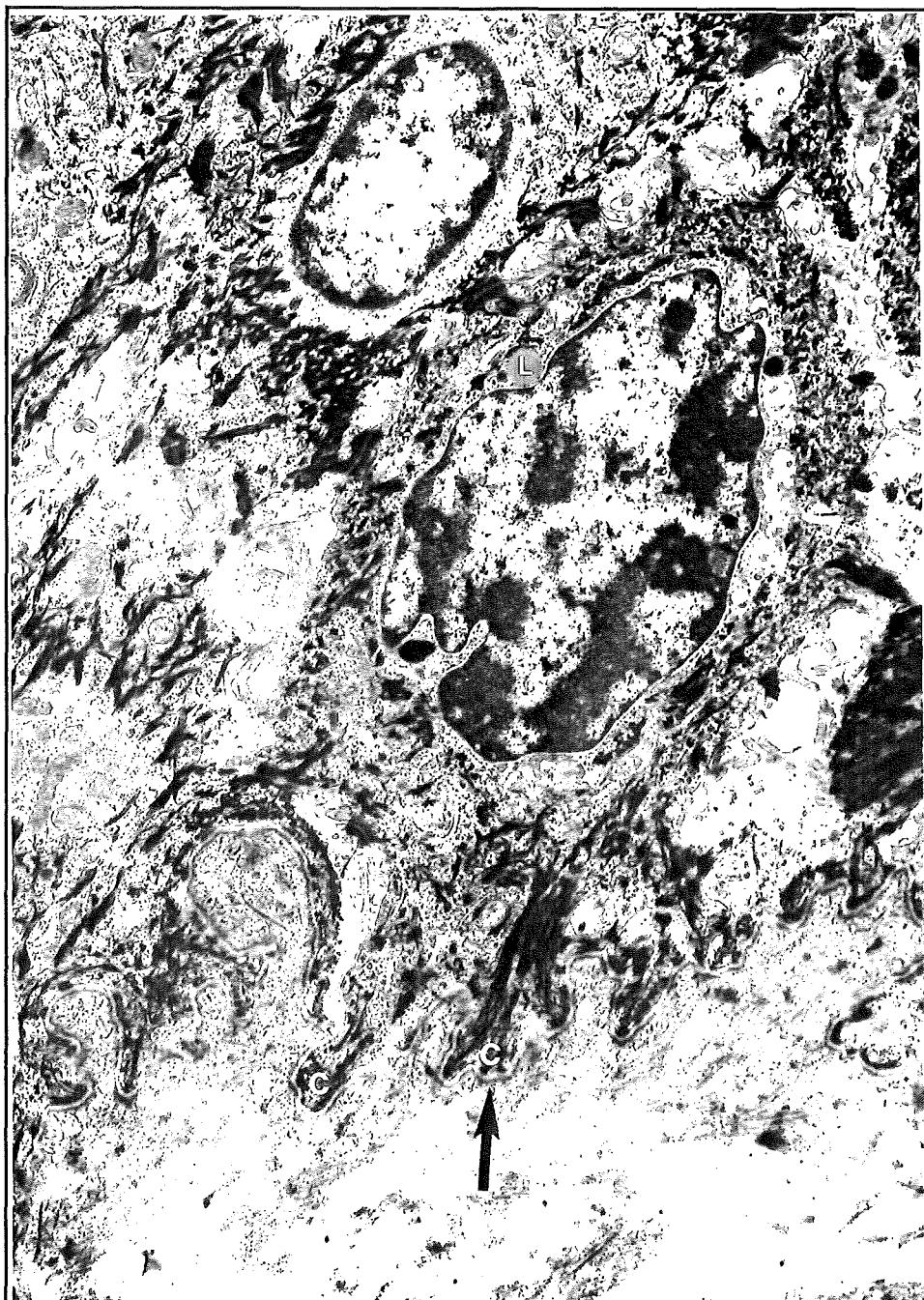


Figure 4.46 3-week-old calf. Stratum basale. The basal plasma membrane is thrown into cytoplasmic processes (c) which are lined by basal lamina (arrow). The basale cells contain tonofilaments, mitochondria and occasional lipid droplets (L). TEM. X 12,000.

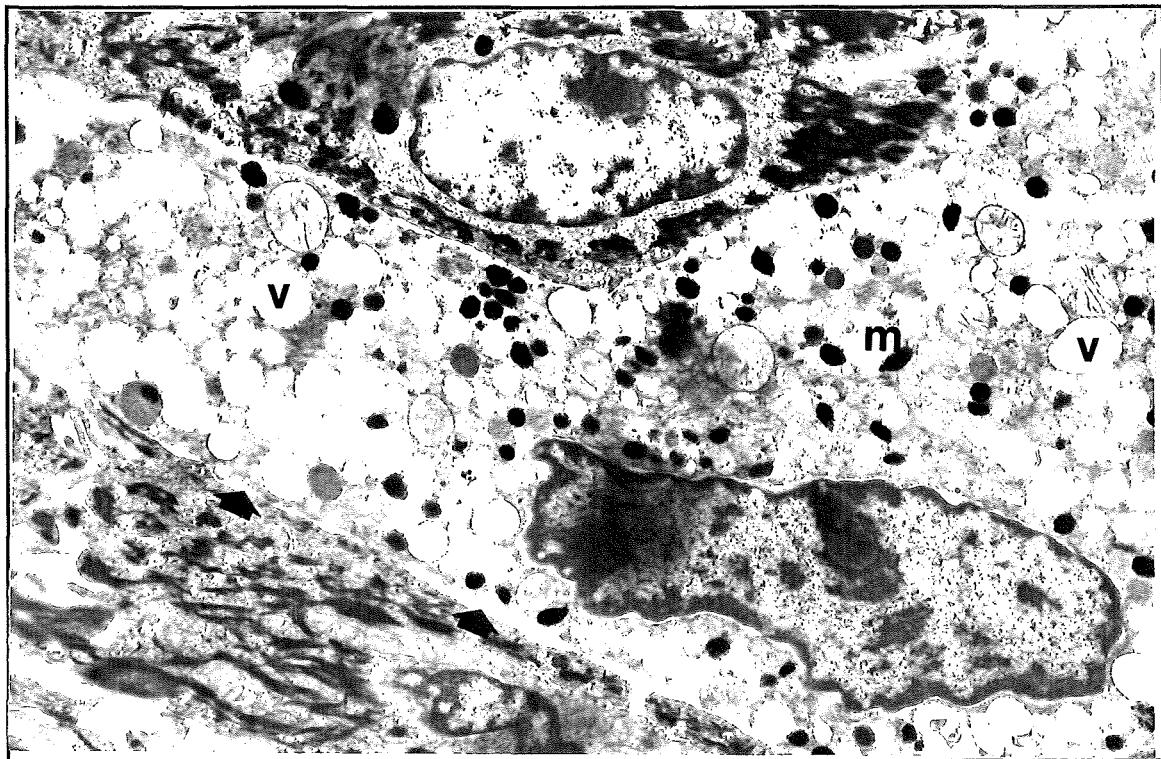


Figure 4.47 4-week-old calf. The melanocyte (m) contains numerous vacuoles (v) and melanosomes. A distinct intercellular space (arrows), uninterrupted by desmosomes, separates the melanocyte from neighbouring keratinocytes. TEM. X 10,000.

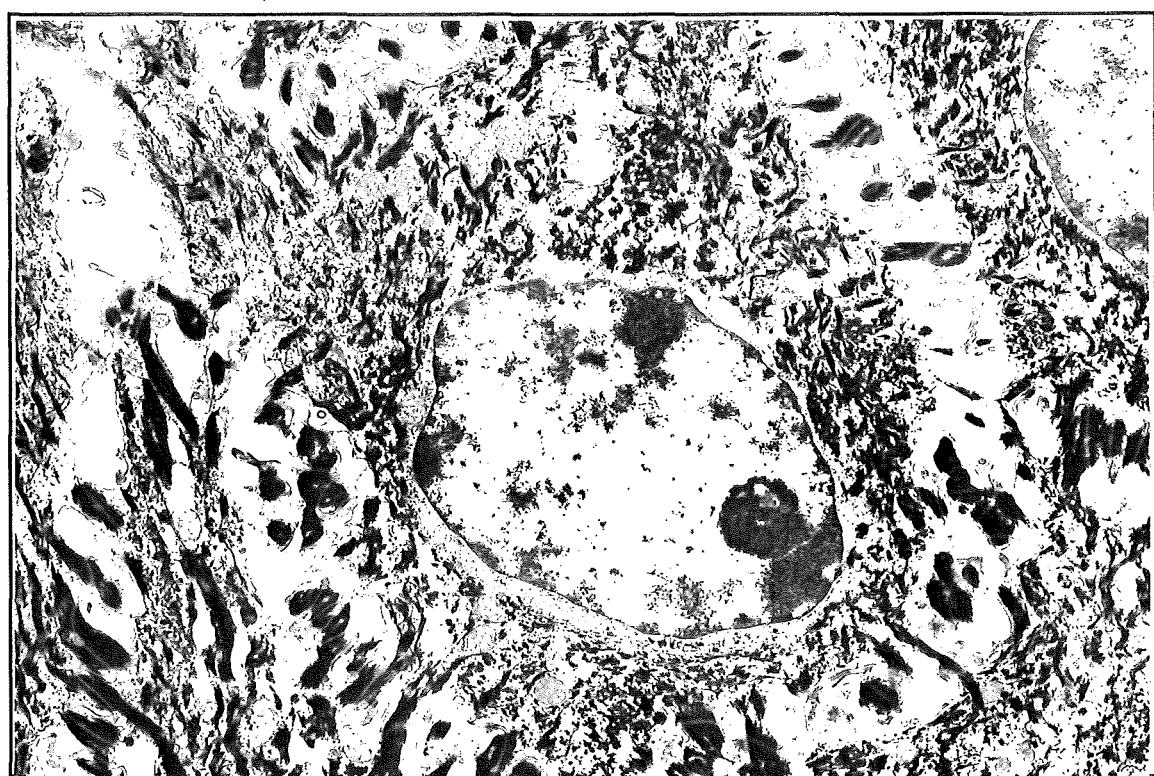


Figure 4.48 3-week-old calf. Stratum spinosum cell. The cytoplasm is filled with tonofilaments and a few mitochondria. Numerous desmosomes link adjacent spinosal cells. TEM. X 8,000.



Figure 4.49 3-week-old calf. Several layers of cells are present in the stratum granulosum, a layer which is characterized by the presence of large keratohyalin granules (arrow). TEM. X 5,400.

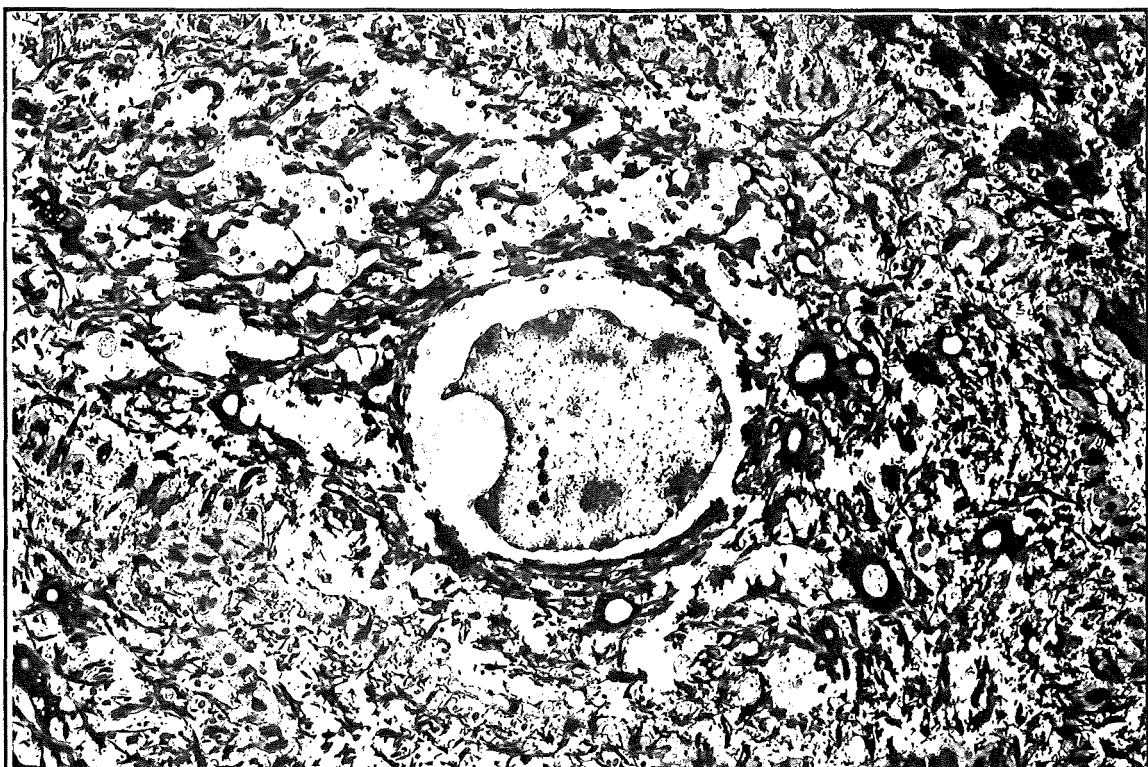


Figure 4.50 3-week-old calf. Granulosum cell with degenerating cellular organelles. Although a few desmosomes are still prominent, most of the plasma membrane is discontinuous. TEM. X 5,400.

CHAPTER 5

**THE PRE-NATAL AND POST-NATAL
DEVELOPMENT OF THE CORNUAL PROCESS
IN THE BOVINE AND CAPRINE: A STUDY
USING HISTOLOGY, RADIOGRAPHY AND
ALIZARIN-RED-S BONE STAINING**

INTRODUCTION

Horned ruminants are sub-divided into four families according to the form of horn present (Geist, 1966; Grzimek, 1972). The first grouping, Cervidae, contains deer which shed their bony antlers annually. The second group, Giraffidae, consists of animals whose horns contain bony cores covered by hairy skin. The third grouping, Antilocapridae, contains pronghorns whose horns are formed by bony elements covered by branched horny sheaths. The horny sheaths are shed annually. The fourth grouping, Bovidae, contains ungulates whose horns consist of bony cores covered by permanent, unbranched horny sheaths.

Horned male ruminants use their horns to fend off other males and thus gain priority access to the females in the herd (Kitchener, 1988). On the other hand the females use their horns to fight off predators (Kitchener, 1985). In addition horn fights are used to defend territory and to determine social ranking (Kitchener, 1987). Horn fights exert immense mechanical forces on the horn base (Kitchener, 1985; 1987; 1988). It would appear that the sensory innervation of the horn limits the amount of force the animal places on the horn base during fights.

The horns of domestic ruminants are supported by a bony core known as the cornual process. The cornual processes of domestic ruminants differ from the antlers of deer in their development and permanence. The cornual process develops by the process of intramembranous ossification, in which bone is formed without the presence of a cartilage model, whilst antlers develop by endochondral ossification in which bone is formed within cartilage (Banks, 1993; Li and Suttie, 1994, 1996; Goss, 1995). The cornual process of domestic animals is a permanent structure, whilst antlers are shed annually. The bony support of giraffe horn differs from both domestic ruminants and deer in that it develops from the ossification of fibrocartilage (Ganey *et al.*, 1990).

Although the cornual process in domestic ruminants is known to develop by intramembranous ossification there is still controversy surrounding the origin of the bone. Although most anatomical textbooks (Grasse, 1948; Sisson and Grossman, 1976; Barone, 1986; Banks, 1993; Dyce *et al.*, 1996) describe the cornual process as arising directly from the frontal bone, Ganey *et al.* (1990) reported the presence of a separate centre of ossification for the cornual process in bovine foetuses. The aim of this study was therefore to investigate the presence of a separate centre of ossification for the cornual process in pre-natal and post-natal calves and kids.

LITERATURE REVIEW

At least three theories on the development of the cornual process in cattle and goats exist. After performing transplantation experiments on post-natal calves and kids, Dove (1935) proposed that the bone forming the cornual process originated from three sources. The frontal bone and deep dermis formed the base of the cornual process, whilst the tip of the process developed in the reticular dermis. Whereas Dove (1935) observed this latter separate centre of ossification in post-natal animals, Ganey *et al.* (1990) reported the presence of an osseous disc over the suture separating the frontal and parietal bone in bovine foetuses. Ganey *et al.* (1990) proposed that the cornual process developed as a separate centre of ossification which eventually fused to the underlying frontal bone before birth. In addition George (1955) and Schummer *et al.* (1981) have described the cornual process as being a separate ossification centre in the early development of sheep and goats. George (1955) reported the separate ossification centre as being cartilaginous. Despite the reports of a separate ossification centre for the cornual process, the most widely accepted current theory is that the cornual process develops as an extension of

the frontal bone (Sisson and Grossman, 1976; Habel, 1981).

MATERIALS AND METHODS

ANIMALS

Sixty-four bovine foetuses were obtained from the City of Glasgow abattoir. The foetuses were of Friesian and Aryshire breeds. Details of the bovine foetuses used for histology, Alizarin-red-S staining and radiography are shown in Tables 5.1, 5.2 and 5.3.

Eight Friesian calves, aged between 2 days and 4 weeks, and ten Cashmere kids, aged between 10 hours and 8 weeks were used in the histological investigation of post-natal cornual process development.

LITERATURE REVIEW OF TECHNIQUES

BONE STAINING WITH ALIZARIN-RED-S

Bone staining with Alizarin-red-S has four basic steps: fixation; maceration; staining, and clearing.

Fixation

Early descriptions of the Alizarin-red-S procedure used 95% alcohol to fix the tissues prior to staining (Dawson, 1926). Since then, modification of the technique has resulted in various fixatives being used. Williams (1941) preferred to use 10% formalin as it prevented soft tissue from being totally macerated. However, the use of formalin adversely affected the quality of bone staining. Burdi (1965) solved this problem by using a mixture of formalin, alcohol and

acetic acid to fix the tissues. A more recent technique uses unfixed tissue without affecting the quality of staining. The use of unfixed tissue allows the procedure to be completed in 2 hours instead of the 5 hours required when the tissue is fixed (Kimmel and Trammell, 1981).

Maceration

During the maceration stage, connective tissue and muscle are broken down, resulting in a transparent layer above the bone (Hood and Neill, 1948). The maceration stage, using potassium hydroxide, has remained unchanged over the years, apart from varying the amount of time a specimen remains in the solution (McLeod, 1980). Maceration needs to be closely monitored to prevent the total disintegration of the foetus (McLeod, 1980).

Staining

Techniques have been described in which Alizarin-red-S is used to stain developing bone in foetuses (Hood and Neill, 1948). However, in specimens where both bone and cartilage are present double-staining techniques using Alizarin-red-S in combination with either toluidine blue (Williams, 1941; Burdi, 1965) or alcian blue (Wasserug, 1976) are employed. Earlier methods required bone and cartilage to be stained in separate steps. However, the staining procedure has been modified to allow bone and cartilage to be stained in one step, which has resulted in a decrease in the amount of staining time required (Wasserug, 1976; Kimmel and Trammell, 1981). Alcian blue and toluidine blue effectively stain cartilage when used in conjunction with Alizarin-red-S. However, they do not successfully stain cartilage in specimens which have been previously stained with Alizarin-red-S and stored in glycerin. Recent advances in the Alizarin-red-S technique, however have now made it possible to stain

cartilage in specimens which had previously been stained with Alizarin-red-S and stored in glycerin (Boardman *et al.*, 1984). This technique involves placing specimens in 3% acetic acid, resulting in stain being transferred from bone to adjacent cartilage. The cartilage stains purple whilst the bone remains red.

Clearing

The staining of bone with Alizarin-red-S results in a small amount of dye in the soft tissues surrounding the bone. Clearing removes such excess stain from the soft tissues (Dawson, 1926). Specimens are cleared in potassium hydroxide and glycerin (Hood and Neill, 1948). Clearing is facilitated by the use of trypsin, in addition to potassium hydroxide (Dingerkus and Uhler, 1977; Kelly and Bryden, 1983;)

RADIOGRAPHY

The early use of radiography to study ossification centres in foetuses was often criticised due to a lack of contrast between bone and soft tissue (Meyer and O'Rahilly, 1958). The use of heavy metals, such as silver or lead, to replace calcium in bone led to an increase in the radiopacity of bone, which made it possible to accurately detect centres of ossification (Hodges, 1953). Radiography has since been used to study bone development in the human (O'Rahilly and Gardner, 1972), pig (Meyer and O'Rahilly, 1958; Wrathall *et al.*, 1974), cat (Smith, 1968; Boyd, 1968), cattle (Lindsay, 1969^a, 1969^b, 1972 ; Lindsay *et al.*, 1969; Gjesdal, 1969), sheep (Richardson *et al.*, 1976) and red deer (Wenham *et al.*, 1986).

TECHNIQUES

LIGHT MICROSCOPY

The horn bud and underlying bone were fixed in buffered neutral formalin for 5 days, after which the bone was decalcified in ethylene-diamine-tetra-acetic acid. The tissues were then processed in an Histokinette and embedded in paraffin wax. 5 μ m sections were cut and stained with Masson's trichrome stain.

BONE STAINING USING ALIZARIN-RED-S

Seventeen bovine foetal heads were stained using the procedure described by Hood and Neill (1948). The heads were fixed for 3 days in 95% alcohol. They were then partially macerated in 2% potassium hydroxide for 4 days, by which time the bones were visible through the transparent skin. The specimens were then stained in a 1:10,000 aqueous solution of Alizarin-red-S for 4 hours. They were then placed in the following clearing solution for 2 days:

2% potassium hydroxide	200ml
0,2% formalin	200ml
glycerin	200ml

After 2 days the specimens were transferred to the following solution for one day:

2% potassium hydroxide	100ml
glycerin	400ml

The heads were then placed in pure glycerin which was changed after 2 days.

RADIOGRAPHY

Crown-rump measurements were taken to estimate the ages of the fifty-nine bovine foetuses used in this study (Table 5.3). The heads of nine of the foetuses were removed and sectioned in half. It was assumed that bone development was identical on both sides of the head. One half of the head was processed for histology or stained with Alizarin-red-S, whilst the other half was fixed in 95% alcohol for two days. The foetuses were then transferred to a 0.5% silver nitrate solution for three days, during which the silver partially replaced the calcium in the bone. The foetuses were radiographed to give a lateral projection. The frontal bone was then removed and radiographed to give a dorsoventral projection.

Table 5.1 Details of bovine foetuses used in the light microscopic study.

Estimated gestational age (days)	Crown-rump length (mm)	Sex
91	165	female
112	245	male
117	260	female
125	290	female
146	350	female
154	390	male
159	430	female
169	480	male
182	530	female
198	580	female
204	605	female
206	625	male

Table 5.2 Details of bovine foetuses stained with Alizarin-red-S.

Estimated gestational age (days)	Crown-rump length (mm)	Sex
70	95	male
80	130	female
81	132	male
84	143	female
85	146	female
87	153	male
91	165	male
93	170	male
95	175	male
99	185	male
103	205	female
104	210	male
106	220	female
108	230	female
109	235	female
112	245	female
168	475	female

TABLE 5.3. Details of bovine foetuses radiographed after silver impregnation.

Estimated gestational age (days)	Crown-rump length (mm)	Sex	Estimated gestational age (days)	Crown-rump length (mm)	Sex
58	61	male	149	365	female
64	77	male	152	380	female
66	83	female	153	387	male
67	86	male	156	408	female
69	91	female	159	430	male
70	95	male	163	450	male
71	102	male	165	460	female
73	104	female	167	470	female
76	115	male	169	480	male
78	122	male	178	514	male
81	132	male	181	525	male
86	150	male	203	600	female
89	160	male	204	605	female
93	170	male	206	625	female
95	175	male	207	630	male
97	180	male	211	670	female
100	188	male	235	760	male
101	195	female	239	780	male
102	198	female	246	823	male
103	205	female			
104	210	male			
105	215	female			
106	220	female			
109	235	female			
110	239	male			
112	245	female			
113	250	male			
117	260	female			
118	265	female			
119	268	female			
120	270	male			
122	280	female			
124	286	male			
125	290	male			
127	300	male			
128	305	female			
132	315	male			
135	318	male			
139	325	female			
141	330	male			

RESULTS

PRE-NATAL CORNUAL PROCESS DEVELOPMENT

58-80 DAYS OF GESTATION

Alizarin-red-S stained bone

At 70 days of gestation two Alizarin-red-S stained rectangular frontal bones were present over the dorsal region of the head, caudal to the orbit. The frontal bones consisted of bone spicules which radiated caudodorsally from the dorsal borders of the orbital rims. A pair of small elongated parietal bones were positioned caudal to the frontal bones. The parietal bone spicules radiated outwards from a strongly stained centre. The developing horn bud was situated over the caudal edge of the frontal bone, adjacent to an unstained area which separated the frontal and parietal bones. In the 80-day-old foetus a few pale-staining bone spicules were present in the previously unstained region between the frontal and parietal bones. The horn bud was situated more caudally due to the growth and increasing convexity of the frontal bone.

Radiography

At 58 days of gestation the frontal bones were seen as reticular, radiopaque areas extending caudodorsally from the orbital rims (Figure 5.1). The parietal bones were oval-shaped and were situated at the caudal edges of the frontal bones. The horn bud was not visible in the 58-day-old foetus.

As the foetuses matured, the frontal bones became more convex. In the 78-day-old foetus the dome-shaped frontal bones were separated from the parietal bones by a wide radiolucent area (Figure 5.2). In this foetus the horn bud was situated close to the caudal border of the frontal bone.

81-111 DAYS OF GESTATION

Histology

In the 91-day-old foetus the bone underlying the horn bud consisted of isolated islands of immature trabecular bone which contained numerous large osteocytes. The trabeculae forming the bone were orientated with their longitudinal axes parallel to the horn bud surface. New bone formation, characterized by clumps of basophilic osteoblasts, occurred at the tips of the trabeculae.

Alizarin-red-S stained bone

By 81 days of gestation numerous red-stained bone spicules were identified in the area between the frontal and parietal bone, linking the bony plates of the two bones. In addition, the frontal bone extended further into the area separating it from the parietal bone. As in the previous age group, the horn bud was situated over the caudal region of the frontal bone. By 95 days of gestation the margins of the frontal and parietal bones overlapped (Figure 5.3).

Radiography

By 93 days of gestation, radiopaque bone spicules from the caudal margin of the frontal bone, and the rostral margin of the parietal bone had invaded the radiolucent area between the two bones. As a result of the ingrowing bone plates, the radiolucent area between the two bones had narrowed. At 97 days of gestation the frontal and parietal bones overlapped. Later, at 103 days of gestation, a wider area of the two bones had overlapped, resulting in the absence of the radiolucent area which had previously existed between the two bones (Figure 5.4). The caudal region of the horn bud was

situated over the overlapping bones.

112-142 DAYS OF GESTATION

Histology

In the 112-day-old foetus the bone underlying most of the horn bud consisted of a main bone plate, with the frontoparietal suture situated under the caudal region of the horn bud. Histological sections in this area revealed the presence of isolated islands of developing bone below the main bone mass (Figure 5.5).

Alizarin-red-S stained bone

In all the foetuses studied the frontal and parietal bones overlapped. In some foetuses the horn bud was placed solely over the frontal bone, whilst in others it was situated partly over the frontoparietal suture.

Radiography

By this stage of development, the margins of the frontal and parietal bones had overlapped extensively beneath the caudal region of the horn bud.

143-173 DAYS OF GESTATION

Histology

In this age group most of the horn bud was situated over the frontoparietal suture, hence the presence of two bone plates below the horn bud in most histological sections. The two bone plates were formed by immature trabeculae orientated parallel to the epidermal surface. Loose, irregular connective tissue

containing blood vessels and nerve fascicles separated the two bone plates. As the foetuses matured the bone plates increased in thickness, but remained separated by connective tissue.

Alizarin-red-S-stained bone

Both frontal and parietal bones were composed of well-developed red-stained bone spicules. The frontal and parietal bones overlapped to a greater extent compared to the previous age group. In the 168-day-old foetus, the caudal region of the horn bud was positioned over the area of bone overlap (Figure 5.6).

Radiography

In all the foetuses studied, the developing horn bud was located over the frontoparietal suture.

174-246 DAYS OF GESTATION

Histology

During this period new bone spicule formation was limited to the upper regions of the frontal and parietal bones. At 204 days of gestation bone formation was more active in the frontal bone where large lacunae were observed. The deeper bone plate consisted of well-developed trabeculae with small lacunae. In the 206-day-old foetus the bone underlying the horn bud had become dome-shaped, indicating the beginning of cornual process development. New bone formation occurred at the apex of the cornual process.

Radiography

A radiopaque line marked the cranial extent of the parietal bone. The reticular pattern of the frontal and parietal bones had been replaced by dense radiopaque bone.

POST-NATAL CORNUAL PROCESS DEVELOPMENT

CALVES

HISTOLOGY

2 days to 1 week old

In both calves a convex cornual process, formed by both the frontal and parietal bones, extended into the dermis underlying the horn bud. In the 2-day-old calf the frontoparietal suture was situated close to the base of the cornual process, whilst in the 1-week-old calf the suture was situated close to the apex of the cornual process (Figure 5.7 A&B). The periosteum was thickest over the apex of the cornual process.

2-3 weeks old

The horn bud was situated over the frontoparietal suture in three of the four animals. In one of the 2-week-old calves the cornual process was an extension of only the frontal bone. In this animal no suture was visible in the developing cornual process. A thick periosteum was present over the top of the cornual process in all the calves. The bony trabeculae below this area were orientated with their longitudinal axes perpendicular to the epidermal surface. New bone formation appeared to be most active at the tips of the apical trabeculae.

4 week old

The dorsal half of the cornual process was composed of vertically arranged trabeculae, whilst the base was composed of horizontally placed trabeculae. The cornual process was an irregular-shaped structure with a thick cellular periosteum.

KIDSHISTOLOGY10 hour old

The cornual process was an extension of the frontal bone. The deep trabeculae of the cornual process were orientated parallel to the epidermal surface, whilst the superficial trabeculae were arranged perpendicular to the epidermal surface.

2-week-old kid

As in the 10-hour-old kid, the cornual process was an extension of the frontal bone. However, situated above the fibrous periosteum of the cornual process was a wedge-shaped ossification area (Figure 5.8). This osteogenic region contained bone matrix lined with basophilic osteoblasts. Numerous osteocytes enclosed in large lacunae were observed within the bone matrix. The immature bone was surrounded by presumptive osteoprogenitor cells possessing oblong-shaped nuclei and scant, basophilic cytoplasm (Figure 5.9). Cells with lobated nuclei and eosinophilic cytoplasm, thought to be eosinophils, were seen within the ossification centre. Few collagen bundles were observed amongst the osteoprogenitor cells, although collagen bundles were observed within the bone matrix.

4-8 week old kids

In one of the 4-week-old kids, a flat-topped cornual process extended into the dermis. Above the flattened surface of the cornual process was an ossification centre surrounded by presumptive osteoprogenitor cells (Figure 5.10 A&B). The ossification centre contained large osteocytes enclosed in a bone matrix. A fibrous periosteum separated the cornual process from this ossification centre. In the 6 to 8-week-old kids studied, the cornual process arose as an extension of the frontal bone.

DISCUSSION

In the youngest foetus stained with alizarin-red-S the horn bud was situated over the caudal region of the frontal bone. In older foetuses the frontal bone and the parietal bone overlapped resulting in the presence of two bone layers below the caudal region of the horn bud. Likewise, in the post-natal calves both the frontal and parietal bones formed the cornual process. Thus, the results of the present study have shown that both the frontal and parietal bones are involved in cornual process development in the majority of pre-natal and post-natal calves. This finding is contrary to the accepted view of the cornual process arising as an outgrowth from only the frontal bone (Sisson and Grossman, 1976). The development of the cornual process in the present study resembled that of the bony core in giraffe horn, in which both frontal and parietal bones form the bony core of the horn (Ganey *et al.*, 1990).

In histological sections of bovine foetal horn Ganey *et al.* (1990) noted the presence of a disc-shaped ossification centre above the frontoparietal suture, which they suggested was the developing cornual process. The disc-shaped ossification centre was seen in a single bovine foetus in the third trimester of

gestation. Furthermore George (1955) noted the presence of a cartilaginous centre in the horn region of foetal and neo-natal sheep. However, in the present study none of the radiographs or Alizarin-red-S-stained heads of bovine foetuses between 58 and 246 days of gestation revealed the presence of a separate ossification centre for the cornual process. Likewise, none of the histological sections of horn buds, from foetuses aged between 91 and 206 days of gestation, demonstrated the presence of a separate ossification centre above the frontoparietal suture.

Although a separate centre of ossification for the cornual process was not recognized in the pre-natal and post-natal bovine specimens, separate ossification centres, for part of the cornual process, were seen in two kids. In both kids the ossification centres were located in the reticular dermis above a well-developed cornual process which arose as an outgrowth of the frontal bone. A thick periosteum separated the cornual process from the ossification centre. It is assumed that this ossification centre would have eventually fused with the underlying cornual process through the periosteum. In the present study ossification centres in the reticular dermis were not seen in the 6 to 8-week-old kids, suggesting that the fusion of the bones occurred between 4 and 6 weeks of age. However, since separate ossification centres were seen in only two kids it is not clear whether the cornual processes of all kids develop in this manner. The separate ossification centres observed in the two kids are in agreement with findings of transplantation studies carried out by Dove (1935). Dove (1935) stated that the tip of the cornual process develops as a separate ossification centre in the reticular dermis whilst the base of the process arises from the frontal bone and deep dermis. As in the present study the ossification centres observed by Dove (1935) were separated from the cornual process by a fibrous periosteum. Despite the report by Dove (1935) the widely accepted view is that the cornual process in kids is an outgrowth from the frontal process (Sisson and

Grossman, 1976).

The ossification centres observed in the present study were composed of numerous osteoprogenitor cells surrounding an area of immature bone. The immature bone was characterized by the presence of numerous osteocytes in large lacunae. In addition the fibrous frame on which bone matrix was being deposited was still visible. This description of immature bone is in agreement with reports of bone formation by intramembranous ossification (Banks, 1993).

In the current study the osteoprogenitor cells surrounding the newly-formed bone resembled the cells observed in the cellular periosteum. However, since a thick fibrous periosteum separated the two areas containing osteoprogenitor cells, the source of the cells forming the separate ossification centre is unclear. It is possible that the cells could have migrated from the cellular periosteum into the reticular dermis where they increased in number by cell division. The other possibility is that the osteoprogenitor cells were derived from fibroblasts in the reticular dermis. It has been reported that fibroblasts are able to differentiate into osteoprogenitor cells when given the appropriate stimulation (Roberts *et al.*, 1982; Banks, 1993).

SUMMARY

It was noted in the present study that in some calves the cornual process was an outgrowth from both the frontal and parietal bones.

A separate centre of ossification for the cornual process was not observed in any of the pre-natal and post-natal calves studied using histology, radiography and Alizarin-red-S bone staining. However, a separate ossification centre, for part of the cornual process, was observed in two out of the eight kids studied histologically. The ossification centres, were in both cases, separated from the main cornual process by a periosteum.

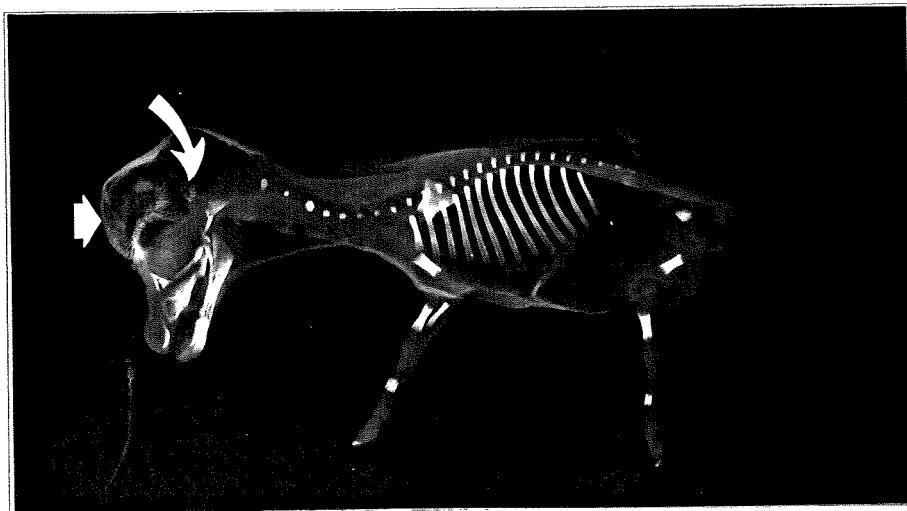


Figure 5.1 Radiograph, lateral projection. 58-day-old bovine foetus. The radiopaque spicules forming the frontal bone (short arrow) extend caudodorsally from the orbital rim. The parietal bone (long arrow) is situated at the caudal edge of the frontal bone. A horn bud was not apparent in this foetus.



Figure 5.2 Radiograph. Lateral projection. 78-day-old bovine foetus. A wide radiolucent area (arrow) separates the frontal and parietal bones. The horn bud (circle) is situated close to the caudal margin of the frontal bone.

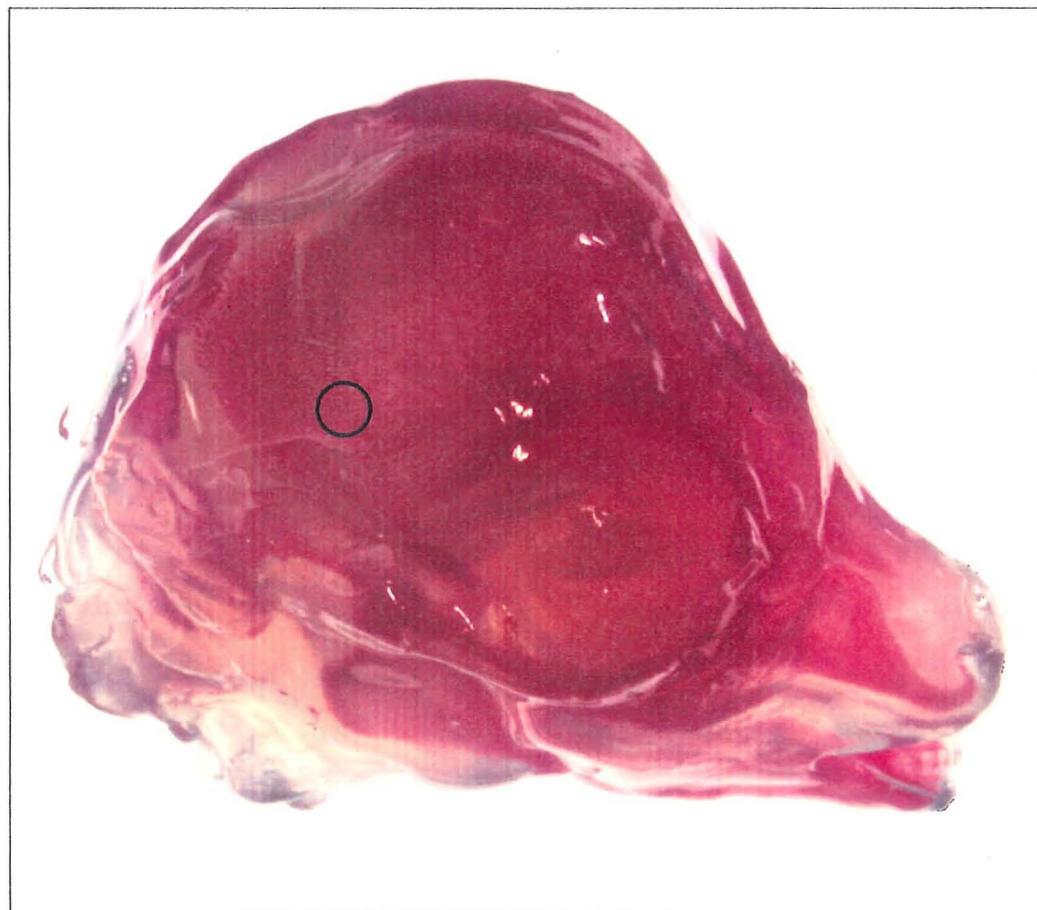


Figure 5.3 95-day-old bovine foetus. The caudal border of the frontal bone extends over the rostral margin of the parietal bone. The horn bud (circle) is situated over the overlapping frontal and parietal bones. Alizarin-red-S stain.

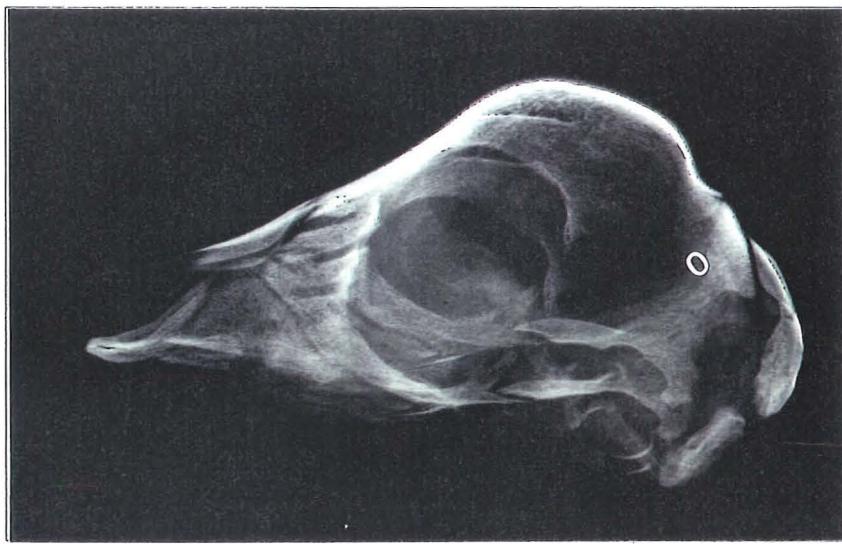


Figure 5.4 Radiograph. Lateral projection. 103-day-old bovine foetus. The frontal and parietal bones have overlapped completely in the area underlying the caudal region of the horn bud (circle).

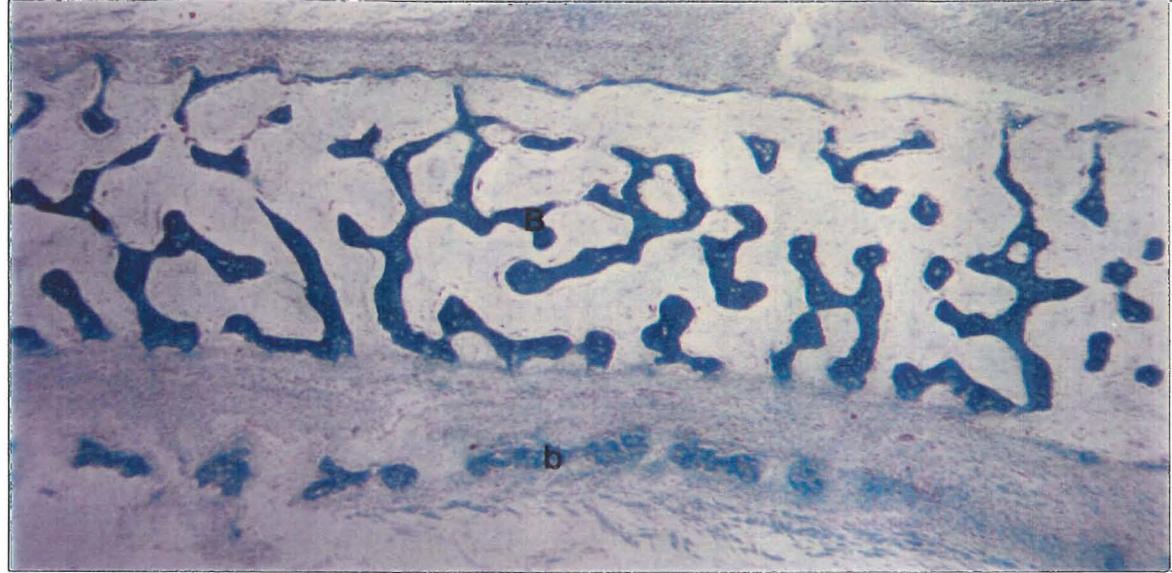


Figure 5.5 112-day-old bovine foetus. Section of developing skull in the horn region. Areas of new bone (b) characterized by the presence of numerous osteocytes are seen below a thick bone mass (B) in the horn bud area. Masson's trichrome. X 75.

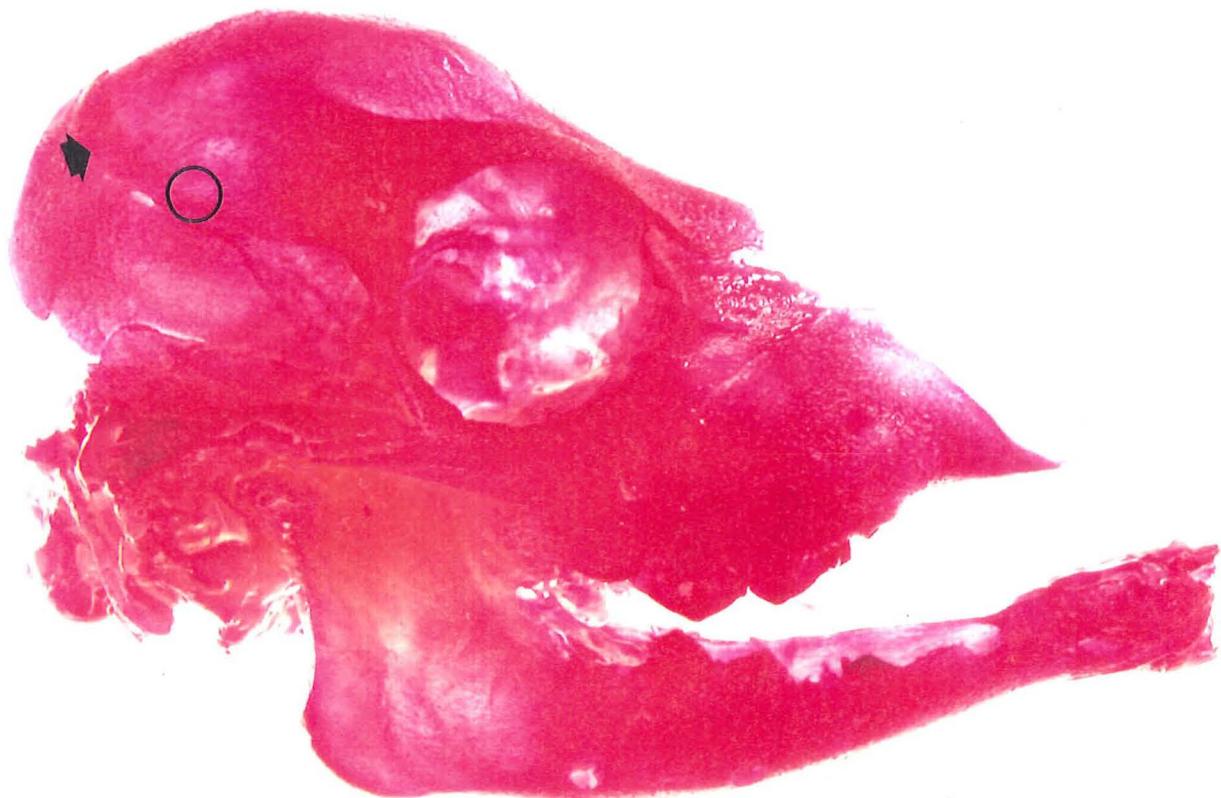


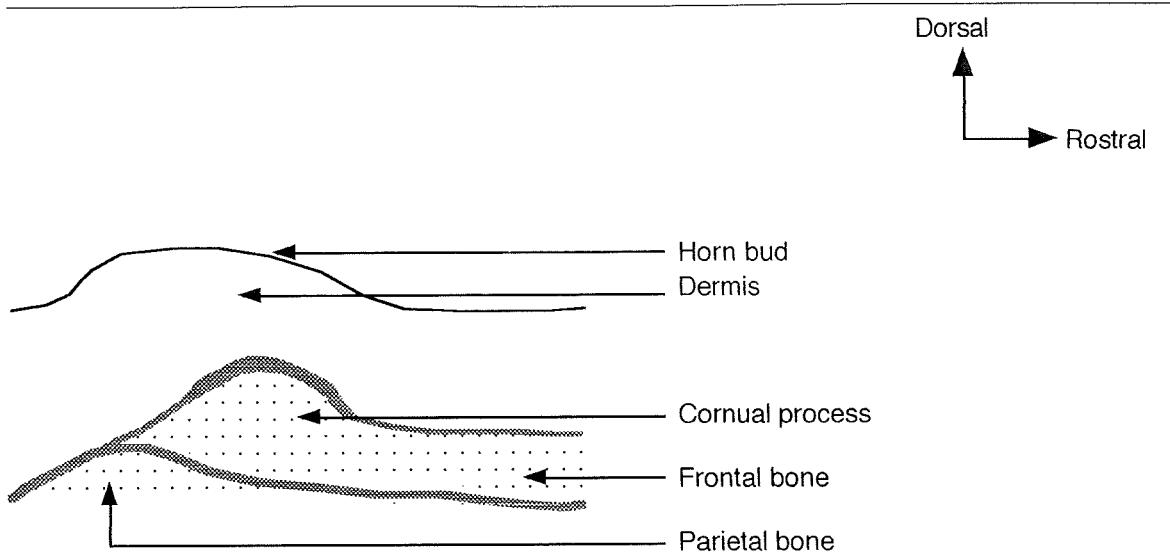
Figure 5.6 168-day-old bovine foetus. The frontal bone covers the rostral region of the parietal bone. The caudal region of the horn bud (circle) covers the overlapping bones (arrowhead). Alizarin-red-S- stain.



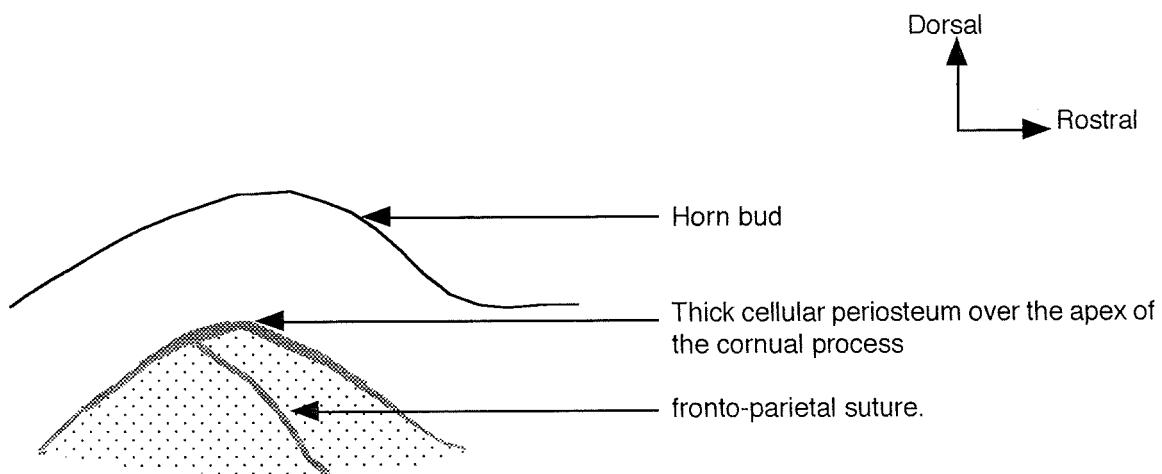
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A.



B.

Figure 5.7 Graphic representations of the cornual process. **A.** 2-day-old calf. The fronto-parietal suture is situated near the base of the cornual process.

B. 1-week-old calf. The fronto-parietal suture is positioned close to the apex of the cornual process.

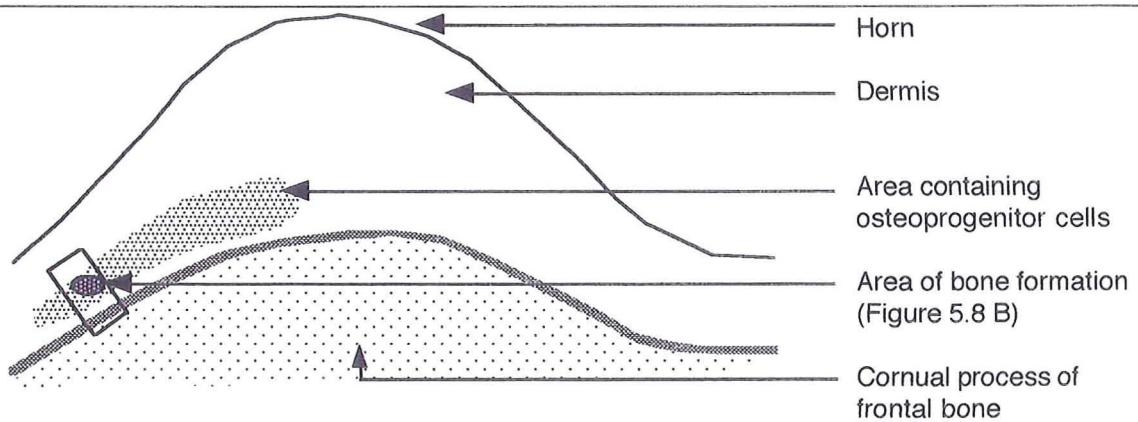
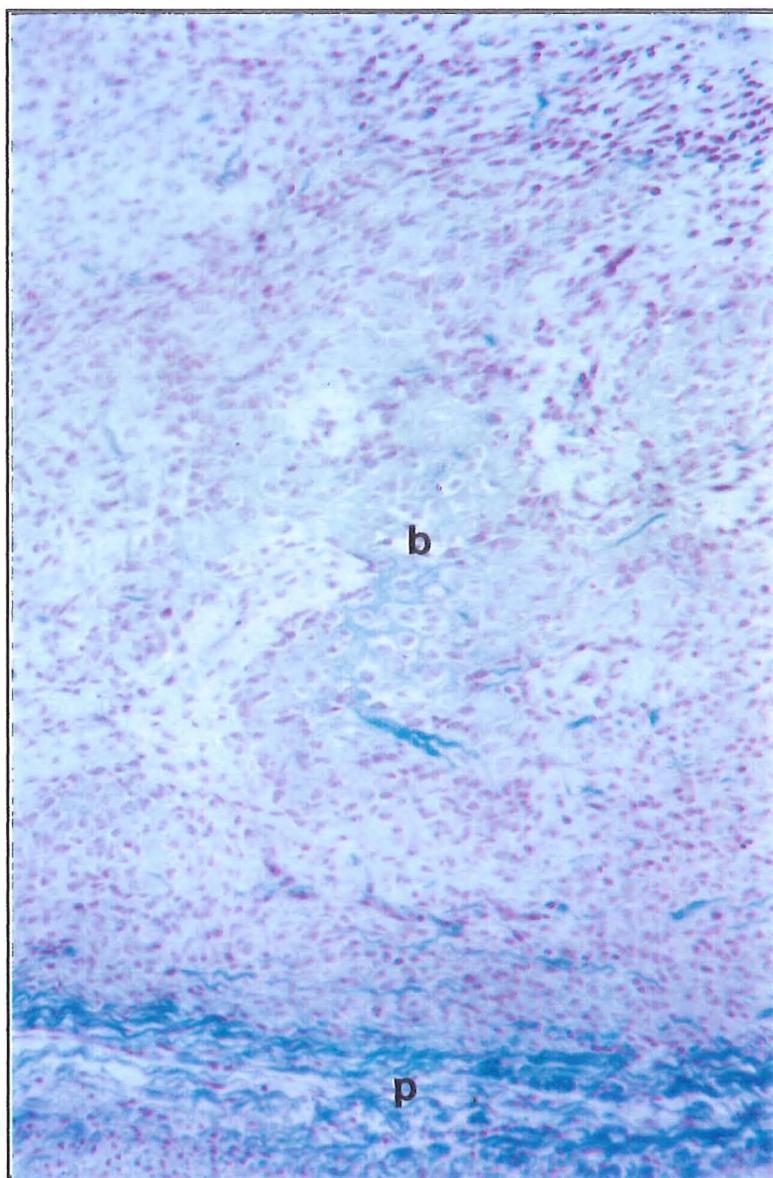
**A.**

Figure 5.8 2-week-old kid. **A.** Line drawing to show the location of the separate ossification centre.
B. The ossification centre contained a locus of immature bone (b). A layer of fibrous periosteum (P) separated the ossification centre from the underlying cornual process. Masson's trichrome. X 187,5.

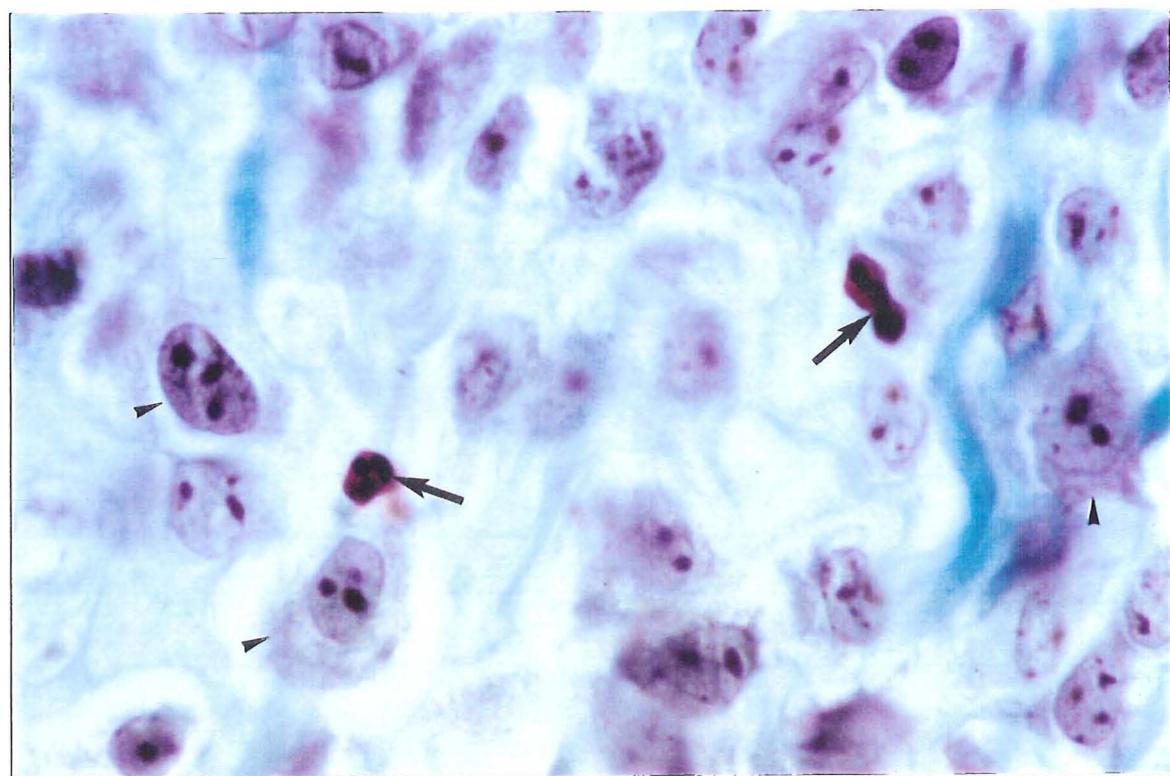


Figure 5.9 2-week-old kid. The ossification centre was composed of numerous osteoprogenitor cells (arrowheads) with oblong-shaped nuclei enclosed in basophilic cytoplasm. Cells with lobated nuclei and eosinophilic cytoplasm (arrows) were seen amongst the osteoprogenitor cells. Masson's trichrome. X 1,875.

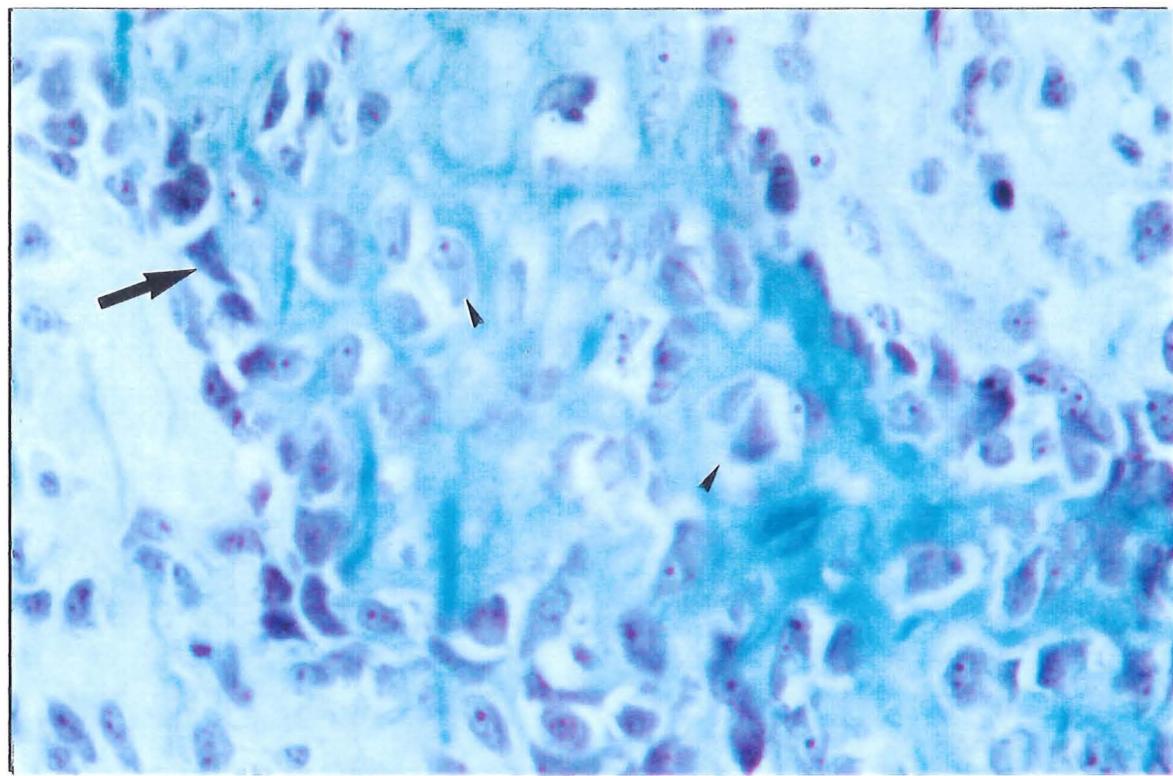
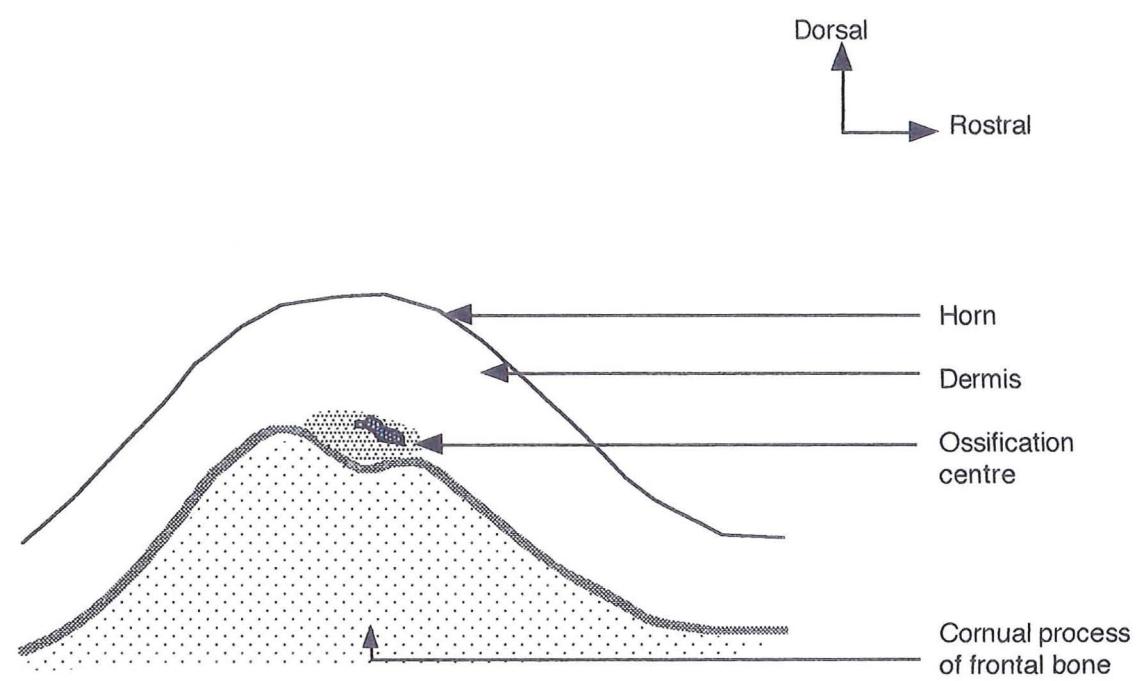


Figure 5.10 4-week-old kid. **A**. Line drawing showing the location of the separate ossification centre in relation to the cornual process.

B. The bone situated within the ossification centre is composed of osteocytes (arrowheads) trapped in bone matrix. A row of basophilic osteoblasts (arrow) line the bone matrix. Masson's trichrome. X 750.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION AND CONCLUSIONS

There are published reports which suggest that some cattle and goats show signs of pain during dehorning, even after the *R.zygomaticotemporalis* has been blocked (Wheat, 1950; Mitchell, 1966). This anaesthetic failure is thought to be due to the presence of additional nerves supplying the horn base. The *N.frontalis*, *N.infratrochlearis*, *N.sinuum frontarium* and *Nn.cervicales* have been mentioned as nerves which occasionally innervate the horn base (Blin, 1960; Lauwers and De Vos, 1966). Of these nerves, the *N.sinuum frontarium* has received the least attention in the literature. The first objective of this study was therefore to provide more information on the contribution of the *N.sinuum frontarium* to horn innervation, in addition to providing a systematic description of the other nerves which may innervate the horn base. The latter part of the objective was achieved by grossly tracing the branches of the *N.ophthalmicus* and *Nn.cervicales* in the vicinity of the horn. For this part of the investigation twenty-five cattle and twenty-four goat heads were dissected. The cattle ranged in age from 2 days to 4 years, whilst the goats ranged in age from 10 hours to 6 years.

Gross dissections confirmed the *R.zygomaticotemporalis* as the main nerve supplying the horn base in cattle. Furthermore it was established that the *N.infratrochlearis* and *N.frontalis* sent branches to the horn base in a few cattle. However, no branches from the *Nn.cervicales* could be traced to the horn base in any of the animals studied.

In addition to gross dissections, the neurotracer Fluorogold was used to identify the nerves cut during dehorning. The neurotracing technique involved the application of Fluorogold to the horn base after dehorning. Fluorogold was taken up by the cut ends of sectioned nerves and then transported in a retrograde direction along the nerves. After four to six weeks the location of the Fluorogold was revealed by studying sections of nerves under the fluorescent microscope. Two calves aged 5 and 6 weeks and two 15-month-old heifers were

used in the Fluorogold study, which appears to be the first time such a procedure has been attempted on domestic ruminants.

In agreement with the observations of the gross dissections, retrograde tracing using Fluorogold showed that the *R.zygomaticotemporalis* is the main nerve supplying the horn base in cattle. In addition a lesser contribution from the *N.infratrocLEARIS* and *N.frontalis* was established. A major result of the retrograde tracing technique was the observation, for the first time in cattle, that branches from the *R.zygomaticotemporalis* on one side of the head occasionally cross over the crown to innervate the horn on the other side of the head.

Although Fluorogold was demonstrated in the *R.zygomaticotemporalis*, no Fluorogold was identified in the *N.sinuum frontalium*, thus indicating that this nerve did not extend as far as the cornual diverticulum in the animals studied. The absence of *N.sinuum frontalium* branches in young cattle was supported by an histological study of the cornual diverticular lining, which showed a lack of nerve fascicles in cattle less than 2 years of age. More significantly, it was shown that branches of the *N.sinuum frontalium* were present in the cornual diverticuli of cattle over 2 years of age. This has serious implications for the dehorning of cattle over 2 years of age in which blocking the *R.zygomaticotemporalis* will not completely desensitize the horn base. These animals will need to be dehorned under general anaesthesia or will alternatively require a nerve block, such as the standard eye block (Peterson, 1950), which desensitizes all the branches of the *N.ophthalmicus*.

The situation in goats is similar to that observed in cattle. Branches of the *N.sinuum frontalium* were noted in the cornual diverticuli of animals over 4-years-old. As in cattle, the presence of nerves in the cornual diverticuli of animals may cause incomplete anaesthesia if only the *R.zygomaticotemporalis* and *N.infratrocLEARIS* are blocked.

Although the Animals (Anaesthetics) Act states that adult cattle and goats

should not be dehorned without the use of local anaesthesia, the law regarding the disbudding of calves and kids is not as strict. This appears to be due to the general belief that neonatal animals feel a minimum amount of pain as the horn is poorly developed. However, although the horn is poorly developed no studies have previously been carried out to determine whether the sensory innervation of the horn is poorly developed soon after birth. It was therefore thought worthwhile to carry out a study on the sensory innervation of the horn bud in calves and kids.

The sensory innervation of the horn was investigated using silver staining, immunohistochemical techniques, and transmission electron microscopy. Silver staining was, until recently, the most commonly used method of nerve fibre demonstration. However silver staining has the disadvantage of being unreliable and unable, in many cases, to stain fine nerve fibres. This was the case in the present study where silver staining produced unsatisfactory results. As a result immunohistochemistry was relied upon to demonstrate the sensory receptors present in the horn bud of calves and kids. The immunohistochemical study utilized neurofilament and S-100 antibodies, which identified nerve fibres and Schwann or lamellar cell cytoplasm respectively. In this way it was possible to trace the course of intraepidermal nerves, as well as appreciate the inner lamellar structure of bulbous corpuscles.

A major feature of the immunohistochemistry investigation was the demonstration for the first time of the presence of sensory receptors in the horn bud of calves and kids. It was revealed that the post-natal horn bud in calves and kids has numerous free nerve endings which are generally accepted as being nociceptors. This finding emphasises the sensitivity of the horn bud to the tissue damage that takes place during disbudding.

Apart from the observation of free nerve endings, it was interesting to note the presence of well-developed sensory receptors in the horn bud of even the

youngest animals. It was noted that bulbous corpuscles were the commonest type of corpuscular receptor encountered. Using the immunohistochemical technique the structural features of the bulbous corpuscles were clearly visible. A prominent feature of the bulbous corpuscle was the presence of an inner lamellar core which was demonstrated using an antibody against the S-100 protein located in the lamellar cell cytoplasm. Bulbous corpuscles stained in this manner were seen to have a central unstained region which was thought to indicate the location of an axon. This central location of the axon was confirmed by using an antibody against the neurofilament protein known to be present in nerve fibres.

As few studies have been carried out to determine the ultrastructural features of bulbous corpuscles identified using immunohistochemistry, it was considered worthwhile to carry out such an investigation on the corpuscles identified in the present study. Ultrastructural studies clearly showed that the mass of S-100 immunoreactive cytoplasm surrounding the central axon was formed by regularly placed lamellae.

In addition to bulbous corpuscles, Ruffini corpuscles, perifollicular terminals, Merkel cells and intraepidermal nerves were identified using both immunohistochemistry and transmission electron microscopy. The Ruffini corpuscles and perifollicular terminals were associated with hair follicles which underwent degeneration as the horn bud developed. Although present for only a short while post-natally, both the Ruffini corpuscles and perifollicular terminals were well-developed in the neonatal animals. The Ruffini corpuscles consisted of circularly arranged nerve fibres which were located in the connective tissue sheath of the hair follicle. The nerve fibres were accompanied by Schwann cells and septal cells, the latter of which separated the nerve fibres and adjacent collagen fibres into compartments. As with the Ruffini corpuscles, immunohistochemistry was able to show the structure of the perifollicular

terminals associated with hair follicles in the horn region. The study using both neurofilament and S-100 proteins emphasised the palisade arrangement of the perifollicular terminals around the hair follicles.

As no studies appear to have been carried out on the degeneration of the nerves surrounding hair follicles in the horn region, it was considered worthwhile to use both S-100 and neurofilament antibodies to follow the degeneration of the glial and neural components of these sensory receptors. It was interesting to note that in calves S-100 immunoreactivity of the Ruffini corpuscles was lost before neurofilament immunoreactivity. This was not the case in kids, where S-100 immunoreactivity was retained after neurofilament immunoreactivity had been lost.

Merkel cells and intraepidermal nerves were identified as the neural components in the epidermis. Immunohistochemistry using neurofilament antibodies was able to demonstrate both the Merkel cells and intraepidermal nerves. In addition the neurofilament antibodies labelled the expanded nerves which terminated on the Merkel cells. In this way the association of the Merkel cell with nerve fibres was clearly visible at the light microscopic level.

The following chapter was devoted to the development of the horn bud in pre-natal bovine foetuses, as well as in post-natal calves and kids. This work was stimulated by the need to know more about horn bud development, in order to devise ways of disrupting horn bud formation and thus remove the need for dehorning. To this end the development of the epidermal and dermal regions of the horn bud were studied using histochemistry, scanning electron microscopy and transmission electron microscopy.

Histological examination of bovine foetuses showed the stratum basale in the horn bud region to be composed of between one and four layers of cuboidal to columnar cells. The stratum intermedium in the horn bud region, in common with the adjacent skin, contained large round cells with apically-placed nuclei

and cytoplasm containing tonofilaments as well as glycogen. Amongst the intermedium cells were a few Langerhans cells which lacked tonofilaments. Overlying the stratum intermedium was a periderm layer composed of cells with voluminous central regions and attenuated lateral regions. A major finding of the scanning electron microscope study was the presence of protrusions arising from the periderm cells in the groove surrounding the horn bud. Such protrusions had previously only been noted in wide areas of skin in human foetuses. A further feature of note in the periderm of the horn bud region was the presence of glycogen-filled cells which interdigitated with electron-lucent periderm cells. The presence of large amounts of glycogen in the periderm cells of the horn bud was confirmed by histochemistry.

The final section of this work involved an investigation of the development of the cornual process in calves and kids. This study was stimulated by conflicting reports on the origin of the cornual process. Although the widely accepted view is that of the cornual process originating from the frontal bone, several researchers report the presence of a separate centre of ossification for the cornual process in calves, lambs and kids. As so little information is available on the development of the cornual process in pre-natal and post-natal domestic ruminants, it was considered worthwhile to carry out such a study using histology, radiography and Alizarin-red-S bone staining which are the most commonly employed techniques to demonstrate bone development.

This investigation clearly showed that both the frontal and parietal bones contributed towards the formation of the cornual process in the majority of calves, whereas only the frontal bone formed the cornual process in kids. A separate centre of ossification for the cornual process was not observed in any of the calves studied. However, histological sections of the horn bud in two kids revealed a separate ossification centre for part of the cornual process. In both cases the separate ossification centres were located in the reticular dermis

above a dome-shaped cornual process which arose from the frontal bone.

In conclusion the present investigation on the innervation and development of the horn has shown that, in addition to the *R.zygomaticotemporalis*, branches from the *N.infratrochlearis* and *N.frontalis* may innervate the horn base in cattle. Furthermore, branches from the *N.sinuum frontale* innervate the cornual diverticuli in mature cattle and goats. This latter finding emphasises the need to dehorn animals as soon after birth as possible, when the cornual diverticulum is absent and the cornual process is poorly developed. However, the disbudding of neonatal calves and kids should be performed under local anaesthesia, as it has been shown in the present study that the horn bud is well innervated at this stage.

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