THE POSTNATAL MATURATION OF
THE ORGAN OF CORTI AND STRIA VASCULARIS
IN THE GERBILS.

MARK SOUTER.

A thesis presented for the degree of Ph. D. in the
Institute of Laryngology and Otology,
University College London,
University of London.

JUNE 1996.
This thesis describes the postnatal maturation of the peripheral sensory epithelium of hearing, the organ of Corti, and the stria vascularis in gerbils. Morphological studies of the developing organ of Corti from 2 days after birth (DAB) to 20 DAB were carried out using a combination of techniques including freeze fracture; light microscopy; transmission and scanning electron microscopy. Measurements of the responses of the cochlea to acoustic stimulation were carried out from 10 DAB onward, together with measurements of the endocochlear potential (EP). The principal findings presented in this thesis are 1) whilst sensory cell maturation proceeded with a base to apex gradient, the maturation of the supporting cells and basilar membrane began in the middle turn, followed by the basal turn and finally the apical turn; 2) the basilar membrane did not attain mature thickness until 18 DAB; 3) The apicalmost tight junctional region around the neck of outer hair cells (OHC) had a mature conformation by 8 DAB, whilst the basalmost adherens region did not reach maturity until 16 DAB; 4) Intramembrane protein particle (IMP) density on the lateral membrane of OHCs reached 87% of mature values by 12 DAB, whilst the first complete subsurface cisternal layer was not present until 10 DAB. Pillar structures connecting the outermost cisternal layer to the plasma membrane did not appear until 10-12 DAB; 5) The inner hair cell (IHC) apical junctional region region matured before that of the OHC; 6) The IMP density on the lateral membrane of IHCs reached a peak at 12 DAB before declining to almost mature values by 18 DAB. From 4 DAB onward, large IMPs were found organised in rows. Plaque structures were first seen in small numbers at 8 DAB and only small numbers were found at 16 DAB; 7) Gap and tight junctional specialisations of the basal cells of the stria vascularis were found to mature with a time course similar to the onset and maturation of the EP,
suggesting these cells play a major role in the establishment and maintenance of the EP.

The findings are discussed in relation to current opinion on the role of the outer hair cells in maintaining cochlear sensitivity and frequency selectivity and models of the mechanism of production of the endocochlear potential.
ACKNOWLEDGEMENTS.

Thanks to Andy Forge for discussion, encouragement and support, Professor Tony Wright for "non-scientific" assistance and Graham Nevill for section cutting and his work in the dark room. Special thanks to Liz, Jo and Chloe for putting up with me.
TABLE OF CONTENTS.

TITLE PAGE 1
ABSTRACT 2
ACKNOWLEDGEMENTS 3
TABLE OF CONTENTS 4
LIST OF FIGURES 9
LIST OF TABLES 11

CHAPTER 1. INTRODUCTION. 13
1.1 Anatomy and physiology 13
1.2 The organ of Corti 15
1.3 Inner and outer hair cells 17
1.4 Innervation 20
   A. The afferent innervation 20
   B. The efferent innervation 22
   C. Efferent responses to stimulation and possible roles 25
1.5 The responses of the cochlea to acoustic stimulation 26
1.6 Otoacoustic emissions 29
1.7 The role of OHCs in cochlear mechanics 31
1.8 The OHC and motility 32
1.9 The stria vascularis 35
   A. Marginal cells 35
   B. Intermediate cells 36
   C. Basal cells 37
   D. Capillaries 37
   E. Models of EP generation 38
1.10 Embryology and maturation of the organ of Corti

A. Formation of the otocyst
B. Morphogenesis of the cochlea
C. The receptor epithelium: The organ of Corti

CHAPTER 2. METHODS

2.1 Establishment and maintenance of the gerbil colony

2.2 Preparation of specimens for structural studies
2.3 Dissection and fixation

2.4 Processing for microscopy and freeze fracture

A. TEM and light microscopy
B. Scanning electron microscopy
C. Freeze fracture

2.5 Quantitative data: The organ of Corti

2.6 Physiological response measurement

A. Anaesthesia
B. Surgical procedures
C. Measurement of CM and CAP
D. Measurement of SFOAEs

2.7 Acoustic stimulus generation and delivery

A. Selection of stimulus frequency
B. Acoustic stimulus presentation

CHAPTER 3. RESULTS

3.1 Physiological response maturation

3.1.1 CM and CAP

3.1.2 SFOAEs

3.2 Structural maturation
3.2.1 The organ of Corti 76

A. Sensory cell maturation 76
B. Supporting cells: A middle turn outward gradient 77
C. The body of the organ of Corti 79
D. Inner spiral sulcus and tectorial membrane 79
E. Pillar cells 81
F. Basilar membrane 84

3.2.2 The outer hair cell 86

A. The mature OHC 86
B. The maturation of the OHC: The apical surface 88
C. Junction formation 89
D. The lateral wall: Sub-surface cisternal network 91
E. The lateral wall: Intra-membrane particles 93
F. Basal membrane 94

3.2.3 The inner hair cell 95

A. The mature IHC 95
B. The maturation of the IHC: The apical surface 96
C. Junction formation 97
D. The lateral membrane 99

3.3 Labelling of the tight junctional protein ZO-1 101 in the reticular lamina 101

3.3.1 Introduction 101

3.3.2 Methods 101

3.3.3 Results 102

3.4 The postnatal maturation of the stria vascularis 103

3.4.1 Introduction 103
3.4.2 Methods

A. Morphological studies  
B. Na\(^+\)-K\(^+\) ATPase reactivity  
C. Recording of endocochlear potential
   i) Anaesthesia and surgical procedures  
   ii) Recording of EP

3.4.3 Results

A. The mature stria vascularis  
B. Gross morphological maturation of the stria  
C. Endocochlear potential onset and maturation  
D. Na\(^+\)-K\(^+\) ATPase reactivity  
E. Development of the tight junctions of marginal cells  
F. Tight junctions between adjacent basal cells  
G. Basal cell apical membrane gap junctions

CHAPTER 4. DISCUSSION

4.1 Brief summary of principal findings

4.2 Gross morphological maturation

A. Opening of spaces  
B. Basilar membrane  
C. Maturation of cochlear responses

4.3 OHC maturation

A. The apical membrane  
B. The junctional region  
C. Lateral membrane maturation and OHC motility
   i) Lateral membrane particles  
   ii) Sub-surface cisternae  
   iii) Cytoskeletal pillars  
D. Basal membrane  
E. Summary I

4.4 IHC maturation

A. The apical membrane  
B. The junctional region  
C. The lateral membrane  
D. Summary II
4.5 Junction formation and junctional protein expression 317

A. The inner ear 320

4.6 The stria vascularis 324

A. Na\(^+\)-K\(^+\) ATPase activity 325
B. Marginal cells 326
C. Basal cell tight junctions 327
D. Basal cell gap junctions 328
E. Basal cell junctional maturation and EP onset 328

CHAPTER 5. Summary of findings 330

Conclusion 334

REFERENCES 336
# LIST OF FIGURES.

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic diagram of the mammalian ear</td>
<td>49</td>
</tr>
<tr>
<td>1.2</td>
<td>Schematic cross-section of the cochlea</td>
<td>51</td>
</tr>
<tr>
<td>1.3</td>
<td>Schematic cross-section of the organ of Corti</td>
<td>53</td>
</tr>
<tr>
<td>1.4</td>
<td>Schematic diagram of IHC and OHC</td>
<td>55</td>
</tr>
<tr>
<td>1.5</td>
<td>Innervation of the organ of Corti</td>
<td>57</td>
</tr>
<tr>
<td>1.6</td>
<td>Schematic diagram of the stria vascularis</td>
<td>59</td>
</tr>
<tr>
<td>2.1</td>
<td>Schematic diagram of the gerbil organ of Corti</td>
<td>71</td>
</tr>
<tr>
<td>2.2</td>
<td>Diagram of the arrangement of stimulus tones for SFOAE generation and recording</td>
<td>73</td>
</tr>
<tr>
<td>3.1</td>
<td>CM onset and maturation</td>
<td>116</td>
</tr>
<tr>
<td>3.2</td>
<td>CAP onset and maturation</td>
<td>118</td>
</tr>
<tr>
<td>3.3</td>
<td>SFOAE waveforms</td>
<td>120</td>
</tr>
<tr>
<td>3.4</td>
<td>SFOAE onset and maturation</td>
<td>122</td>
</tr>
<tr>
<td>3.5</td>
<td>SEM views of the maturing organ of Corti</td>
<td>124</td>
</tr>
<tr>
<td>3.6</td>
<td>Organ of Corti width</td>
<td>130</td>
</tr>
<tr>
<td>3.7</td>
<td>Supporting cell head maturation</td>
<td>132</td>
</tr>
<tr>
<td>3.8</td>
<td>Sections through the maturing organ of Corti 2-8 DAB</td>
<td>134</td>
</tr>
<tr>
<td>3.9</td>
<td>Sections through the organ of Corti at 10 DAB</td>
<td>136</td>
</tr>
<tr>
<td>3.10</td>
<td>Sections through the maturing organ of Corti 12-20 DAB</td>
<td>138</td>
</tr>
<tr>
<td>3.11</td>
<td>Tectorial membrane maturation 2-4 DAB</td>
<td>140</td>
</tr>
<tr>
<td>3.12</td>
<td>Tectorial membrane maturation 6-12 DAB</td>
<td>142</td>
</tr>
<tr>
<td>3.13</td>
<td>Tectorial membrane maturation 8 DAB</td>
<td>144</td>
</tr>
<tr>
<td>3.14</td>
<td>Pillar cell orientation 2-8 DAB</td>
<td>146</td>
</tr>
<tr>
<td>3.15</td>
<td>Pillar cell orientation 10-16 DAB</td>
<td>148</td>
</tr>
<tr>
<td>3.16</td>
<td>Opening of the organ of Corti spaces 6 DAB</td>
<td>150</td>
</tr>
<tr>
<td>3.17</td>
<td>Opening of the organ of Corti spaces 8 DAB</td>
<td>152</td>
</tr>
<tr>
<td>3.18</td>
<td>Opening of the organ of Corti spaces 10 DAB</td>
<td>154</td>
</tr>
<tr>
<td>3.19</td>
<td>Pillar cell length</td>
<td>156</td>
</tr>
<tr>
<td>3.20</td>
<td>Adult OHC</td>
<td>158</td>
</tr>
<tr>
<td>3.21</td>
<td>Adult OHC- supporting cell junction</td>
<td>160</td>
</tr>
<tr>
<td>3.22</td>
<td>Adult OHC lateral membrane</td>
<td>162</td>
</tr>
<tr>
<td>3.23</td>
<td>Adult OHC basal membrane</td>
<td>164</td>
</tr>
<tr>
<td>3.24</td>
<td>Apical region of OHCs 2 DAB</td>
<td>166</td>
</tr>
<tr>
<td>3.25</td>
<td>Apical surface of OHCs 2 DAB</td>
<td>168</td>
</tr>
<tr>
<td>3.26</td>
<td>Apical region of OHCs 8-16 DAB</td>
<td>170</td>
</tr>
<tr>
<td>3.27</td>
<td>Formation of OHC junctional region</td>
<td>172</td>
</tr>
<tr>
<td>3.28</td>
<td>Freeze fracture of the OHC junction 2-4 DAB</td>
<td>174</td>
</tr>
<tr>
<td>3.29</td>
<td>Freeze fracture of the OHC junction 8-16 DAB</td>
<td>176</td>
</tr>
<tr>
<td>3.30</td>
<td>OHC lateral membrane: Freeze fracture</td>
<td>178</td>
</tr>
<tr>
<td>3.31</td>
<td>OHC lateral membrane: Freeze fracture</td>
<td>180</td>
</tr>
<tr>
<td>3.32</td>
<td>OHC lateral wall: TEM 6-10 DAB</td>
<td>182</td>
</tr>
<tr>
<td>3.33</td>
<td>OHC lateral wall: TEM 12-16 DAB</td>
<td>184</td>
</tr>
<tr>
<td>3.34</td>
<td>OHC lateral wall: High power TEM views</td>
<td>186</td>
</tr>
<tr>
<td>3.35</td>
<td>OHC lateral membrane: IMP 2-8 DAB</td>
<td>188</td>
</tr>
<tr>
<td>3.36</td>
<td>OHC lateral membrane: IMP 10-16 DAB</td>
<td>190</td>
</tr>
<tr>
<td>3.37</td>
<td>Increase in IMP density during maturation</td>
<td>192</td>
</tr>
</tbody>
</table>
3.38 Basal membrane maturation: 2 DAB
3.39 Basal membrane maturation: 6 DAB
3.40 Basal membrane maturation: 8-12 DAB
3.41 Basal membrane maturation: 16 DAB
3.42 Adult IHC
3.43 Mature IHC; apical junctional region
3.44 Mature IHC; lateral membrane
3.45 Apical surface of maturing IHCs: 2 DAB
3.46 Maturation of IHC apical junction: 2-6 DAB
3.47 Maturation of IHC apical junction: 8-16 DAB
3.48 IHC lateral membrane IMP density
3.49 IHC lateral membrane maturation: 2-6 DAB
3.50 IHC lateral membrane maturation: 6-16 DAB
3.51 IHC lateral membrane maturation: plaques
3.52 ZO-1 labelling of the reticular lamina: 2 DAB
3.53 ZO-1 labelling of the reticular lamina: 8 DAB and Adult
3.54 Schematic diagram of the stria vascularis
3.55 Na⁺-K⁺ ATPase in the mature stria
3.56 Mature stria vascularis: Marginal cells
3.57 Mature stria vascularis: basal cells
3.58 Maturation of the stria: TEM 2-6 DAB
3.59 Maturation of the stria: TEM 12-20 DAB
3.60 EP recording from 20 DAB gerbil
3.61 EP onset and maturation
3.62 Na\textsuperscript{+}-K\textsuperscript{+} ATPase activity: 2- 6 DAB 244
3.63 Na\textsuperscript{+}-K\textsuperscript{+} ATPase activity: 12- 20 DAB 246
3.64 Na\textsuperscript{+}-K\textsuperscript{+} ATPase activity: 6- 20 DAB 248
3.65 Na\textsuperscript{+}-K\textsuperscript{+} ATPase activity: 6- 20 DAB 250
3.66 Marginal cell apical tight junction: 2- 6 DAB 252
3.67 Marginal cell apical tight junction: 8- 12 DAB 254
3.68 Basal cell tight junction maturation: 6- 8 DAB 256
3.69 Basal cell tight junction maturation: 10- 12 DAB 258
3.70 Basal cell tight junction maturation: 16- 20 DAB 260
3.71 Basal cell gap junction maturation: 6 DAB 262
3.72 Basal cell gap junction maturation: 8- 12 DAB 264
3.73 Basal cell gap junction maturation: 18 DAB 266

LIST OF TABLES.
3.1 Organ of Corti width and supporting cell maturation 268
3.2 Basilar membrane maturation 270
3.3 OHC apical junction maturation 272
3.4 OHC IMP and cytoskeletal pillar maturation 274
3.5 IHC apical junction maturation 276
3.6 IHC lateral membrane maturation 278
3.7 Strial marginal cells: apical junction maturation 280
3.8 Strial basal cell tight junction maturation 282
3.9 Strial basal cell gap junction maturation 284
CHAPTER 1.

INTRODUCTION.

Normal auditory perception in mammals depends upon the ability of the peripheral sensory organ of hearing, the cochlea, to perform frequency analysis of complex sounds with a high degree of sensitivity. Studies of the morphological and functional development of the auditory system may provide insights into how these tasks are performed. Determining the morphological changes which occur prior and subsequent to the onset of auditory function may also help in elucidating the factors involved in changes in frequency representation at the periphery during maturation of the organ of Corti and hence the relationship between specific structures of the organ of Corti and cochlear function.

1.1 The anatomy and physiology of the mammalian cochlea.

Reviews of the basic structure and function of the mammalian cochlea can be found in Dallos (1973) and Pickles (1988). The auditory organ of mammals may be considered as consisting of three discrete elements; the outer, middle and inner ear. Figure 1.1 shows a schematic representation of these elements. The outer ear and external auditory meatus serve as a complex resonating cavity attenuating or increasing the sound pressure arriving at the eardrum, the tympanic membrane, in a frequency dependent manner. The middle ear couples the incoming vibrational energy to the auditory organ of the inner ear, the cochlea. Pressure variations at the tympanic membrane are transmitted via a chain of three small bones; the malleus; incus and stapes, to the membrane covered oval window.
of the cochlea.

The mature cochlea is a coiled tube divided into three fluid chambers: scala tympani, scala media and scala vestibuli. It is encased in a spiralling bony capsule which lies at the base of the skull. At the centre of this spiral is the modiolus, through which course the nerve and blood supplies of the cochlea. A schematic cross section of the cochlea is shown in Figure 1.2.

The outermost chambers, scala vestibuli and scala tympani, are separated at the modiolar side by the osseous spiral lamina. Both chambers contain perilymph which is similar in composition to extracellular fluid. The scala vestibuli and scala tympani are continuous through a hole situated at the apical end of the cochlea, the helicotrema. The scala tympani opens to the membrane-covered round window and scala vestibuli opens to the oval window which is covered by the footplate of the stapes and an annular ligament. The central chamber, scala media, is filled with potassium (K⁺)-rich endolymph fluid. The scala media is roughly triangular in cross section, bounded by the thin cells of the Riessner’s membrane which separates the scala media from the scala vestibuli; the stria vascularis which is a multilayer tissue forming the lateral wall of the scala media; and by the basilar membrane, upon which sits the sensory epithelium of the organ of Corti. This forms the floor which separates scala media from scala tympani. The basilar membrane is a fibrous structure attached at the modiolar side to the spiral lamina. It is mechanically tuned, this being a consequence of decreasing stiffness and increasing width toward the apex and is thus able to act as a basic frequency analyser. Vibrations of the stapes displace the fluid of scala vestibuli and sets up a wave-like motion of the basilar
membrane. The displacement reaches a maximum at a point along the basilar membrane determined by the frequency of the stimulus and the dimensions of the basilar membrane. The position of maximal displacement is termed the characteristic place of the stimulus frequency. High frequencies are located toward the base of the cochlea and low frequencies at the apical end. This frequency dependent motion of the basilar membrane provides the stimulus to the sensory cells of the organ of Corti.

1.2 The organ of Corti.

Figure 1.3 shows a diagrammatic cross section of the organ of Corti, the sensory epithelium of the cochlea. The organ of Corti sits on the basilar membrane and comprises the receptor cells with their nerve endings and innervation, and populations of supporting cells. Overlying the organ of Corti is the tectorial membrane which is attached to the limbus on the modiolar side (Lim 1972, Steel 1983). There are two morphologically distinct populations of receptor cells, a single row of inner hair cells (IHC), and typically three rows of outer hair cells (OHC). The organ of Corti is given rigidity by the arrangement of a population of microtubule containing supporting cells, the inner and outer pillar cells (IP and OP respectively), which are oriented to form an arch, the fluid filled tunnel of Corti. Deiters’ cells, which separate the rows of OHCs, also contribute to the rigidity of the organ of Corti. In addition to the tunnel of Corti between the IP and OP cells, fluid filled spaces also surround the OHCs and the phalanges of the Deiters’ cells. These are the perilymph containing spaces of Nuel and are only found in the mature organ of Corti, the immature organ being characterised by the lack of intercellular spaces. Several other morphologically distinct types of supporting cells, Hensens and Claudius, are present toward the stria vascularis side.
The apical surfaces of the sensory and supporting cells are configured in a mosaic-like pattern, with each hair cell separated by a supporting cell. This arrangement is generally referred to as the reticular lamina. By means of intercellular junctional complexes, the reticular lamina maintains the separation between the K⁺-rich endolymph which bathes the apical surface of the organ of Corti and the sodium (Na⁺)-rich perilymph which bathes the cell bodies of the hair cells and the supporting cells. This barrier maintains a positive electrical potential, the endocochlear potential (EP), across it relative to perilymph. The EP recorded in the scala media has a value of around +80-100 millivolts (mv), the precise value varying between species. The EP is thought to be maintained by the stria vascularis and is essential for the normal functioning of the cochlea, as it provides the driving potential for current flow through the hair cells. The total potential difference across the apical membrane of the IHCs is approximately 125 mv, made up of an EP of, for example, 80 mv and an intracellular resting potential of -45 mv (Russell and Sellick 1978), whilst the potential across the apical membrane of OHCs is about 150 mv (80 mv EP + -70 mv OHC intracellular resting potential; Dallos et al 1982, Dallos 1985, Cody and Russell 1985, 1987).

In contrast to the non-stimulus dependent endocochlear potential, several stimulus-evoked electrical potentials can be recorded with electrodes sited close to the organ of Corti and which can be identified as originating from the organ of Corti. The cochlear microphonic (CM) is an a.c. response which follows the waveform of the stimulus and originates in currents flowing through the hair cells. The vast majority of this current originates from the OHCs, by virtue of there being approximately three times as many OHCs as IHCs. This has been demonstrated experimentally, since when OHCs were selectively destroyed
by ototoxic agents the CM was almost totally abolished (Dallos 1973). The summating potential is seen as a base line shift which accompanies the CM and has its origin in d.c. components generated by the hair cells. The gross neural response of the cochlea to acoustic stimulation, the compound action potential (CAP), can also be recorded. This potential represents the summed responses of a large population of cochlear afferent nerves responding to the acoustic stimulus.

1.3 Inner and outer hair cells.

The two types of sensory cell in the organ of Corti, the inner and outer hair cells, differ in their morphologies and innervation patterns (Lim 1986) and are shown in schematic cross section in Figure 1.4. Both IHCs and OHCs have common features with other acousticolateral hair cells, for example vestibular hair cells and the lateral line hair cells of fish and amphibians. Organised rows of stereocilia, which are modified microvilli, emerge from the apical membrane of the hair cells, arranged in a step configuration with the tallest row farthest from the modiolar side. This morphological polarity also confers a functional polarity on the hair cells (see below), and is thought to be determined during development by the kinocilium, which is a true cilium, and which is found on only immature mammalian cochlear hair cells. During development of cochlear hair cells, the tallest row of stereocilia are positioned on the modiolar side of the kinocilium, no stereocilia are found on the outermost region of the hair cells' apical surface. OHC stereocilia are arranged in a "W" pattern whilst those of the IHCs form a shallow arc. Structurally, the stereocilia contain closely packed actin filaments (Flock and Cheung 1977), which confer rigidity. Closely packed actin filaments also form a rootlet which anchors the stereocilium in the cuticular plate, a densely packed matrix of apparently
randomly arranged actin filaments (Hirokawa and Tilney 1982) together with other cytoskeletal elements, for example myosin (Drenckhanhn et al 1985). Fine strands link the lateral walls of each stereocilium to its neighbour so that deflections of the tallest stereocilia cause the bundle to move as a whole. A second group of fine links are present between the apical tips of the shorter stereocilia and the lateral wall of taller row. These may be involved in the transduction process (Pickles et al 1984).

The tallest row of OHC stereocilia are embedded in the underside of the tectorial membrane, whilst the IHC stereocilia are free standing in the endolymph fluid of the subtectorial space. Therefore, the stimulus delivered to the IHC stereocilia is dependent upon the interaction of the tectorial membrane-OHC-basilar membrane complex. Deflections of the stereocilia in the excitatory direction, toward the position occupied by the kinocilium, cause an influx of ions due to a potential gradient across their membranes. This influx creates an intracellular depolarisation, a receptor potential, leading in the case of the IHCs to the release of neurotransmitter at the base of the cell and activation of the afferent nerve fibre.

Below the cuticular plate, the cell body of the sensory cells contains mitochondria and assorted organelles. The cell nucleus is situated centrally in IHCs but more basally in OHCs. Synapses for afferent innervation are found on the basal region of both IHCs and OHCs, with predominantly efferent synapses on the bases of OHCs. The lateral walls of the OHCs in particular exhibit specialisations which may be related to their role in the transduction process and are described below.
Whilst inner hair cells (IHC) are thought to be the mechanoelectrical transducers of acoustic stimuli, the outer hair cells (OHC) have a more complex and at present less clearly understood role. In contrast to IHCs, the OHCs are capable of elongation and contraction in response to direct electrical stimulation (Brownell et al 1985, Ashmore 1987). It is this motile capability with its potential to affect the mechanical response of the organ of Corti, together with the dependence of frequency sensitivity and selectivity on the presence of an healthy population of OHCs which has led to the OHCs becoming the focus of investigations into how the cochlea attains its exquisite response properties.

Morphologically, OHCs are characterised by having a prominent organised system of endoplasmic reticulum, the lateral cisternae, located parallel to the lateral membrane (Gulley and Reese 1977, Forge 1991, Forge et al 1993b). Regularly arranged pillar-like structures are found between the outermost layer of cisternae and the lateral plasma membrane. These pillars are associated with a cortical cytoskeletal lattice organised in a helix around the cell (Holley and Ashmore 1990, Holley et al 1992, Kalinec et al 1992). In addition, freeze fracture replicas show OHC lateral membranes have large numbers of densely packed intramembrane particles (IMP), which represent membrane-intercalated proteins (Gulley and Reese 1977, Forge 1991). These particles have been suggested to be related to the motor elements which drive the motile response of the OHC (Kalinec et al 1992), although there is no direct evidence to support this interpretation. If these IMPs, or a proportion of them, were to be the sites of the putative OHC motor elements, it may be possible to relate the onset of OHC motile activity to the appearance of the IMPS on the lateral membrane. He et al (1994) found motility of gerbil OHCs to be first evident from about 8 DAB onward. The relationship between the onset
of OHC motility and the density of the IMPs on the OHC lateral membrane will be one of the questions addressed in this thesis.

The apical part of the lateral membrane forms tight junctional complexes with the adjacent supporting cells, creating seals that prevent the passive ionic diffusion along intercellular spaces. These junctional complexes help to maintain the endocochlear potential across the apical membrane, as well as providing attachment to the supporting cells. The intercellular junction between hair cells and supporting cells is unusually deep and of complex organisation. The depth appears to correspond to the depth of the cuticular plate on the hair cell side and to the presence of cytoskeletal elements associated with the membrane on the supporting cell side (Henderson et al 1994). The cuticular plate is attached to the membrane by a fine fibrils of actin (Hirokawa and Tilney 1982). It maybe therefore, that the complex intercellular junctions represent a specialised junction through which supporting cell cytoskeletal elements and the cuticular plate are anchored providing a rigid support for the hair cell cuticular plate and stereocilia. The relationship between the complexity of the junction between the sensory and supporting cells and the onset and maturation of EP will be examined in this thesis.

1.4 Innervation.

A schematic diagram showing the afferent and efferent innervation of the organ of Corti is shown in Figure 1.5.

A. *The afferent innervation.*

Both afferent and efferent innervation is found in the mature organ of Corti. The great preponderance of auditory nerve afferents innervate the IHCs (Spoendlin 1972). The cells
of the spiral ganglion form the primary afferent innervation of the cochlea, connecting the hair cells at the periphery with the cochlear nucleus in the brain stem. The spiral ganglion fibres, which are bipolar, have their cell bodies in the modiolus and form two distinct populations termed Type I and Type II which innervate the IHCs and OHCs respectively in the proportion of approximately 9:1 (Spoendlin 1978).

Type I spiral ganglion fibres, which comprise about 90% of the organ of Cortis' afferent innervation and synapse exclusively on IHCs, are myelinated and between 10 -20 μm in diameter (Schwartz et al 1983). They give rise to the peripheral myelinated radial fibres of approximately 3 μm diameter (Kiang et al 1982) which enter the organ of Corti through the habenula perforata, where they lose their myelin, and synapse on the IHC closest to its entry point. Type I fibres exhibit diverging innervation, with a single IHC being contacted by many afferent nerves. Each afferent fibre synapses on a single IHC, each IHC having around 20 fibres forming synapses (Spoendlin 1978). Type I fibres are highly tuned in response to acoustic stimuli. The frequency of the stimulus which elicits firing of the nerve at the lowest stimulus level is termed the characteristic frequency (CF) of the neuron. The fibres respond to tones at their CF with a continuous discharge following an initial delay of 1-3 ms. This delay is a simple function of the frequency of the stimulating tone and position along the basilar membrane which corresponds to the characteristic place of that frequency (Kiang et al 1965, Evans 1972).

Type II spiral ganglion fibres make up the remaining 10% of the afferent innervation, having a smaller diameter than Type I fibres (< 12 μm) and generally lack myelin but contain large numbers of neurofilaments. The peripheral projections of Type II spiral
ganglion fibres form the outer spiral fibres, which are less than 0.5 μm in diameter and unmyelinated. After passing through the habenula perforata, the fibres course over a variable distance, cross the tunnel of Corti before branching and synapsing on up to 10 OHCs (Spoendlin 1972, Kiang et al 1982). In contrast to Type I afferents, the Type II fibres exhibit a converging innervation pattern, with a single afferent fibre synapsing on many sensory cells.

The central projection of the afferent fibres is to the cochlear nucleus. No primary afferent fibres are thought to pass this point. At the level of the cochlear nucleus, the fibre bundle bifurcates into ascending, descending and dorsal populations, which reside in the anteroventral, posteroventral and dorsal cochlear nuclei respectively. At this level (the brain stem) the tonotopic organisation of the central auditory system is evident, the tonotopicity established at the periphery being maintained to higher centres (Cant and Morest 1979, Aitkin 1986).

B. The efferent innervation.

In 1946 Rassmussen described a bilaterally originating centrifugal or efferent innervation of the mammalian cochlea. These fibres had their cell bodies in the superior olivary complex region of the brainstem and projected by way of crossed and uncrossed fibres to the periphery, terminating on the bases of the OHCs or on the radial afferent fibres innervating the IHCs. These efferent fibres have been termed the olivocochlear fibres due to the location of their cell bodies. The precise functional role of these fibres remains a subject of speculation.

Reviews of the anatomy and physiology of the efferent innervation can be found in Klinke and Galley (1974) and Warr et al (1986).
The development of axonal transport labelling techniques has allowed the identification and tracing of the pathways of the olivocochlear neurons from their origins in the brainstem nuclei to termination site. Two main populations of cochlear efferents have been identified, termed lateral and medial (Warr et al 1982, Warr and Guinan 1979, Guinan et al 1983, White and Warr 1983). The former have their cell bodies sited close to or within the lateral superior olivary nucleus (LSO), whereas the medial fibres have cell bodies scattered medially, ventrally and anteriorly to the medial superior olive (MSO). The two populations exhibit differences of morphology and termination site. The cell bodies of LSO originating fibres are unmyelinated and project predominantly to the ipsilateral cochlea and form synapses on the afferent fibres below the IHCs (White and Warr 1983, Robertson 1985). The majority of studies indicate that IHCs are not directly innervated by efferent fibres, although there is some evidence that a very small number of efferent fibres may synapse directly on IHCs (Brown 1985). The neurotransmitter of LSO neurones is unknown although several candidates have been suggested, including GABA (Schwartz et al 1988). MSO originating fibres have predominantly contralateral projections synapsing on the bases of the OHCs. They further differ from LSO neurones in having large numbers of mitochondria and being myelinated. The major neurotransmitter of the medial efferent fibres is thought to be acetylcholine (Bobbin and Konishi 1971, Guth et al 1976) although immunocytochemical studies have suggested the presence of other excitatory amino acid neurotransmitters (Fex et al 1985).

Recordings from single, identified efferent neurones have shown them to be highly tuned and organised tonotopically (Robertson and Gummer 1985, Cody and Johnstone 1982a, Liberman and Brown 1986, Brown 1989). It has been assumed that these fibres are those
originating in the MSO. Fex (1962) found efferent fibres, recorded from at the midline, which had regular spontaneous firing rates, long latencies of 10-30 ms, relatively high thresholds of around 50 dB SPL and low discharge rates of maximally 60 spikes per second to acoustic stimuli. Cody and Johnstone (1982a) demonstrated that these efferent fibres showed sharp tuning and found units which responded optimally to ipsilateral or contralaterally presented acoustic stimulation. Robertson (1984) recorded from histologically identified efferent fibres and found evidence for a tonotopic organisation of the efferent synapse. In a more extensive study, Robertson and Gummer (1985) found a contralateral/ipsilateral preferred response difference of 43/49%, with the remainder being bilaterally activated.

The evidence suggests there exists populations of neurones which are predominantly ipsilaterally or contralaterally activated. Single efferent fibres are frequency selective and innervate a region of cochlea having afferent fibres of similar CF (Robertson and Gummer 1985, Liberman and Brown 1986, Brown 1989). Contralateral sound-evoked effects have their largest effects in the 1-2 kHz region (Warren and Liberman 1988b), with tones of the same frequency as the ipsilateral stimulus being the most effective suppressors up to 1.5 kHz, whilst broad band noise is most effective for ipsilateral tones above this frequency (Liberman 1989). The highest tone-evoked efferent discharge rates are also seen with MOC neurones with CFs near 1.5 kHz (Liberman and Brown 1986). In cats, the suppression of the auditory nerve action potential by broad band noise has been associated with excitability of the efferent neurones, with those subjects showing greater suppression also exhibiting greater efferent fibre excitability (Liberman 1989).
C. Efferent responses to stimulation and possible roles.

The precise functional role of the efferent innervation remains to be adequately defined. One possible role may be in assisting the detection of a functionally important acoustic signal in background noise (Winslow and Sachs 1987) by manipulations of the auditory periphery. Experimental studies, reviewed in detail by Weiderhold (1986), involving electrical stimulation of the efferent fibres in animals, have shown a reduction in auditory nerve fibre activity (Galambos 1956, Wiederhold 1970, Wiederhold and Kiang 1970), increases in CM (Fex 1959, Konishi and Slepian 1970), and changes in the IHC receptor potential (Brown and Nuttall 1984). There is also evidence from reported changes in otoacoustic emissions that electrical stimulation of efferent fibres may affect the mechanical response of the basilar membrane (Mountain 1980, Seigel and Kim 1982, Guinan 1986, Kemp and Souter 1988). Suppression of auditory nerve activity by a contralaterally presented tone or broad band noise has been shown to be mediated by the efferent neurones, mainly the medial fibres, since section of the fibre tract removes the effect (Warren and Liberman 1988a). There is also evidence for a frequency specific effect of contralaterally presented noise, mediated by the efferent innervation, at the level of auditory nerve firing responses (Buno 1978, Cody and Johnstone 1982a, Liberman 1989). Warren and Liberman (1988b) found that auditory nerve fibres could, when stimulated near their characteristic frequency, have their firing rates reduced by the addition of contralateral tones or noise as low as 30 dB SPL.

It has been suggested that the efferent system may have a role in protection from acoustic overstimulation, since studies using guinea pigs found that contralaterally presented sound may protect the ipsilateral cochlea from damage caused by a loud tone (Cody and

Although section of olivocochlear fibres eliminated the suppressive effect of contralaterally presented noise on auditory nerve activity (Liberman 1988, Warren and Liberman 1988a), section of the efferent fibres does not appear to affect normal compound action potential thresholds and amplitudes, nor does it affect masking phenomena in normal hearing guinea pigs (Rajan 1989, Rajan et al 1990). Although Bonfils et al (1986) reported some changes to masking characteristics following efferent tract sectioning, other workers (Rajan et al 1990, Littman et al 1992) have failed to find a similar effect. Levels of otoacoustic distortion products recorded in the meatus are also unchanged following olivocochlear fibre tract section (Littman et al 1992).

1.5 The response of the cochlea to acoustic stimulation.

The classical model of how sound is analysed by the cochlea, as a travelling wave, has its foundation in the work of von Békésy (1960). Using a light microscope and stroboscopic illumination on human cadaver material he observed that the vibrations of the cochlear partition seemed to progress like travelling waves from the base to the apex of the cochlea, resulting in a tonotopic organisation with high frequencies producing the largest vibration at the base of the cochlea and low frequencies at the apex. He further concluded that the cochlea acted as a linear system since increasing the level of the stimulus did not alter the pattern of vibration of the cochlear partition.
It was difficult, however, to reconcile the linear, broadly tuned vibrational response observed in the basilar membrane motion with the sharply tuned responses of the auditory nerve fibres. It was initially thought that the broadly tuned response of the basilar membrane was processed via a highly tuned "second filter" to produce the neural tuning. Evans and Wilson (1975) proposed a second filter interposed between the basilar membrane motion and neural excitation.

Evidence of nonlinear vibration of the basilar membrane was first obtained by Rhode (1971) in squirrel monkey. Measurements showed that the slope on the low frequency side of the travelling wave was shallow in comparison to the sharp cut off on the high frequency side. A clear nonlinearity at low stimulus intensities was observed on the low frequency side which gave the appearance of a sharpening of the travelling wave peak as it approached its characteristic place. Although initially difficult to replicate (Wilson and Johnstone 1975), Rhode (1973) showed that the vibrational responses of the basilar membrane became linear post mortem. This provided an indication that the response properties of the basilar membrane were dependent to some extent on metabolic processes which contributed to the observed nonlinearity.

Russell and Sellick (1978, 1983) recorded intracellular potentials from IHCs in guinea pig and found that the isoamplitude response for the DC component of the receptor potential was "indistinguishable from threshold tuning curves for auditory nerve fibres" (Russell and Sellick 1978). Their observations led them to conclude that this sharp tuning was due to the mechanical properties of the cochlear partition. Subsequent measurements have shown the basilar membrane motion to be nonlinear in cat (Khanna and Leonard
1982), guinea pig (Sellick et al 1982), and chinchilla (Robles et al 1986, Ruggero et al 1992), leading to the conclusion that discrepancies between neural and mechanical tuning data were probably attributable to differences in sensitivity of technique and degree of inflicted trauma (Kelly and Khanna 1984, LePage 1987).

Although the motion of the basilar membrane is now generally held to be nonlinear and highly tuned (Johnstone et al 1986), it has been suggested that the damping of the cochlear partition would be too great to account for the degree of resonance to produce by passive means the experimentally observed sharp tuning of the basilar membrane.

In 1948, Gold suggested that biomechanical energy available within the cochlear partition might be utilised mechanically to counteract the damping. This proposal led to the consideration that there existed some "active" mechanism in the cochlear partition capable of producing mechanical energy at the expense of metabolic energy. Such a mechanism it was subsequently reasoned, could produce the sharply tuned nonlinear behaviour of the basilar membrane vibration.

The description of stimulus evoked cochlear emissions recordable in the ear canal (Kemp 1978) renewed interest in active cochlear mechanics. These "otoacoustic" emissions were interpreted as being the result of the reflection of individual frequency components from their characteristic place on the basilar membrane, suggesting there existed an active mechanism in the cochlear partition capable of producing mechanical energy and "contributing to the enhancement of cochlear mechanical tuning" (Kemp 1979), that is, producing amplification.
1.6 Otoacoustic emissions.

The externally recordable manifestation of this active mechanism takes the form of cochlear emissions of intracochlear origin, first described by Kemp (1978). In his experiments the sound pressure near the tympanic membrane in humans was recorded in response to acoustic click stimuli. Results showed the stimulus to be followed by a delayed oscillatory response. The delay was from 5 to 15 ms which precluded a middle ear effect. These emissions were found to grow with stimulus level only for low intensities, suggesting that the generating mechanism saturated.

The ability of the cochlea to generate acoustic energy recordable in the ear canal was confirmed by the description of spontaneous emissions (Wilson 1980, Zurek 1981). These could be present in the absence of acoustic stimulation, were highly tuned and capable of being suppressed by a tone of slightly differing frequency. This suggested a highly localised generation site, which was confirmed by the finding that the frequencies of the emission often correlated with lesions found in the organ of Corti which corresponded with what would be the frequency place of the emission (Zurek and Clark 1982, Ruggero et al 1983).

A further class of acoustic emission are the distortion products (DPs). These are stimulated emissions at frequencies other than those contained in the stimulus. They consist of the harmonics of the stimulus and, when more than one tone is present, of the combination tones. DPs are found at readily calculated and constant frequencies, conforming to the expression AF1 ± BF2, where A and B are integers and F1 and F2 are the stimulus frequencies.
The characteristics of DPs have been extensively studied. They were first detected in psychoacoustic studies (for example Goldstein and Kiang 1968) and have been shown to be present in auditory nerve discharges at the DP frequency (Kim et al 1979). This auditory nerve response displays the same characteristics as if it were the product of a single acoustic stimulus at a similar intensity. This led to the conclusion that DPs were present in the mechanical vibration of the basilar membrane and propagated to their characteristic place along the cochlear partition in the same manner as a "normal" stimulus, albeit in a reverse direction which results in their being detectable in the ear canal (Kim et al 1979). Reduction of DP amplitude has been described following a variety of cochlear insults; anoxia; ototoxic drugs and noise trauma (Kim et al 1980, Kemp and Brown 1984 for example). Following electrical stimulation of the efferent fibres, complex changes in DP levels recorded in the ear canal have been also been observed (Mountain 1980, Siegel and Kim 1982, Guinan 1986).

A further category of otoacoustic emissions are stimulus frequency otoacoustic emissions (SFOAE). First described in detail by Kemp and Chum (1980), these are the emissions detectable in the ear canal due to the acoustic stimulus frequency. SFOAEs have advantages over click-evoked emissions and DPs in that the emission originates from a single frequency place, the stimulus frequency, and is not the result of complex wave interactions and propagation effects which give rise to DPs. SFOAEs have been studied in human and guinea pig subjects (Kemp and Souter 1988, Kemp et al 1990, Brass and Kemp 1991, 1993, Souter 1995a,b) and found to be an effective means of monitoring intracochlear active mechanisms. By virtue of their frequency specificity and their relative independence from complex wave interactions in their production (Souter 1995a),
SFOAEs were chosen as the probes with which to investigate the effects of morphological maturation on cochlear mechanical responses in this thesis.

Although believed to have their origin in activity of the cochlear mechano-electrical transducer, thought to reside in the OHCs, all classes of OAE are also dependent upon how this activity is coupled into the functioning organ of Corti. Therefore OAEs are influenced by those elements which affect organ of Corti mechanics; the tectorial membrane; basilar membrane and supporting cells. Thus, the maturation of the OAEs during development will be affected by the maturation of the above structural elements. In this thesis the onset and maturation of the SFOAE at specific frequencies will be examined, and correlated with the maturation of structural components of the organ of Corti.

1.7 The role of OHCs in cochlear mechanics.

As noted above, the candidate for the site of the cochlear mechanical amplifier is the OHCs. Experimental manipulations which affect OHCs have been found to also alter neural tuning responses. Loss or extensive damage of the OHCs results in a substantial hearing loss of up to 50 dB (Ryan and Dallos 1975) and the low threshold neural tuning curve tip is lost (for example Kiang et al 1970, Dallos and Harris 1978, Liberman and Dodds 1984). Mechanical tuning has also been shown to be vulnerable following the systemic administration of furosemide. Furosemide is a diuretic which acts on the cochlea by abolishing the EP, thereby reducing the receptor potentials of the IHCs and OHCs. Ruggero and Rich (1991) found that the sound evoked responses of the basilar membrane were linearized accompanied by a loss of sharpness of tuning following furosemide
administration. They concluded that the cochlear amplifier resided in the OHCs, using the large positive EP as an energy source (Ruggero and Rich 1991). Other evidence of the involvement of OHCs in active cochlear mechanical nonlinearity comes from studies of the effect of electrical stimulation of efferent nerve fibres which synapse on the OHCs and the resulting alteration of varying classes of otoacoustic emissions as noted above (Siegel and Kim 1982, Mountain 1980, Guinan 1986, 1990, Kemp and Souter 1988, 1989). These results showed that OHCs could alter the mechanical performance of the organ of Corti and indicated that the changes seen in IHC tuning curves during efferent nerve stimulation (Brown and Nuttall 1984) where the result of OHC mediated influences on the stimulus to the IHCs.

1.8 The OHC and motility.

In its role as an effector or motor element, the OHC, via its coupling with the basilar membrane and tectorial membrane, is thought to provide some form of feedback of energy into the motion of the basilar membrane (for example Kim 1986). This model would require the OHC to be able to perform vibratory movements at frequencies corresponding to that of the stimulus; in the mammalian ear this would be in the order of kilo Hertz (kHz). Studies of isolated OHCs have shown them capable of elongation and contraction in response to direct electrical stimulation (Brownell et al 1985, Ashmore 1987) at frequencies up to 15 kHz (Reuter et al 1992). This response appears mediated via calcium (Ca^{2+}) dependent K^+ channels in the basolateral wall of the OHC (Ashmore and Meech 1986) and does not appear to be dependent upon adenosine triphosphate (ATP); rather it may be determined by the configuration of the plasma membrane and cell cortex under the influence of membrane potential changes (Holley and Ashmore
A second, slower motile response has also been measured in OHCs. When exposed to high K⁺ concentrations, isolated OHCs undergo a slow contraction associated with a depolarisation of the cell membrane (Zenner et al 1985, Zenner 1986). This form of motility is unlikely to be directly involved in influencing the cycle by cycle response of the organ of Corti, although it may have a role in, for example, adjusting the operating position or membrane potential of the OHC, possibly under the influence of the efferent innervation.

The ATP-independent motility appears to have its site of origin in the basolateral membrane of the OHC. Specialisations of the OHC lateral wall, described above, have been proposed as the site of the OHC motor elements. In particular, arrays of intramembrane protein particles (Kalinec et al 1992), coupled to a filamentous lattice running around the OHC cell body by pillar structures (Holley et al 1992) has been suggested as a candidate model. A study of the relationship between the morphological maturation of these features of the OHC lateral membrane and the onset of OHC motility, to be carried out in this thesis, may be useful in identifying potential locations for the postulated OHC motor elements.

In order to influence the transduction process, the motility must feedback in some way to stimulate the stereocilia on the apices of OHC and IHCs, which are thought to be the sites of the ionic transduction channels. The transduction channels themselves are thought to be sited near the tips of the stereocilia (Jaramillo and Hudspeth 1991), with deflection
of the stereocilia in the excitatory direction opening the channels (Hudspeth and Jacobs 1979, Russell et al. 1986) resulting in an influx of positively charged ions (Corey and Hudspeth 1979). As described above, filamentous tip links run from the apex of smaller stereocilia to a density in the lateral wall of the taller stereocilium in front. In the model proposed originally by Pickles et al. (1984), motion in only an excitatory direction would exert a "pull" on the tip link thus "opening" the transduction channel whilst motion in the opposite direction would have the effect of "closing" the channel. Evidence for the tip links being related to the site of the transduction channel comes from experiments which calculate the number of transduction channels as being approximately equal to the number of tip links (Kros et al. 1992) with 1-2 active transduction channels per stereocilium (Hudspeth 1982) and evidence that breakage of the tip links is followed by the irreversible disappearance of mechanotransduction (Assad et al. 1991, Preyer et al. 1995). The model of tip link involvement has been extended to include gating spring models which account for adaptation in saccular hair cells (Shepherd and Corey 1994, Gillespie 1995). The precise identity of the channel protein is not known.

It is now generally held that a motile mechanism, residing in the OHC lateral membrane is responsible for the sensitivity and sharp tuning of the cochlear mechanical response, achieved through interactions between the tectorial membrane-OHC-basilar membrane complex. It is thought that the energy source driving this system is derived from the EP, which is produced and maintained by a metabolically dependent ionic transfer mechanism sited in the stria vascularis.
1.9 The stria vascularis.

The stria vascularis is a vascularised multilayer epithelium which forms the lateral wall of the scala media. Its primary function is active ionic transport to establish and maintain the high positive endocochlear potential (EP) found in the scala media. The first evidence that the stria is responsible for EP production was provided by Tasaki and Spyropoulos (1959), who found that following the draining of endolymph the surface of the stria was the only cochlear tissue which exhibited a positive potential.

A schematic cross section of the stria is shown if Figure 1.6. The stria vascularis is composed of three cell types; marginal cells which line the scala media; intermediate cells which reach neither the apical nor basal aspects of the tissue and basal cells which are elongated and separate the stria from the underlying spiral ligament. The stria also has a dedicated blood supply, provided by capillaries which run within the tissue.

A. Marginal cells.

The epithelial marginal cells line the scala media, their apical surfaces exposed to the endolymph fluid. The apical aspect is hexagonal and adjacent cells are joined by tight junctions around the apical region of the lateral walls. Their basolateral membranes are extensively infolded, with large numbers of mitochondria and Na\(^{+}\)-K\(^{+}\) ATPase localised at high levels at the membrane (Kuijpers and Bonting 1969, Schulte and Adams 1989). Freeze fracture replicas of the membrane reveal large numbers of intramembrane particles (IMP) which represent membrane proteins possibly related to Na\(^{+}\)-K\(^{+}\) ATPase. Kuijpers (1974) studied Na\(^{+}\)-K\(^{+}\) ATPase activity in the developing rat stria and found increasing enzymatic activity to parallel the maturation of the EP. The presence of the enzyme on
marginal cell lateral walls suggested a major role for the marginal cells in the development of EP.

The dark-staining ion transporting cells of the vestibular system are morphologically similar to the marginal cells of the stria (Kimura 1969) and also possess high levels of \( \text{Na}^+\)-\( \text{K}^+ \) ATPase activity (Nakai and Hilding 1968, Spicer et al 1990). There is, however, no vestibular potential equivalent to EP. As they are the only strial cells lining the scala media the marginal cells are ultimately responsible for the maintenance of endolymph, however the extent of their involvement in EP generation is unclear. It is possible that the marginal cells are involved in \( \text{K}^+ \) transport but not necessarily in EP generation. Studies of diuretic and ouabain administration have shown differing effects on EP levels and \( \text{K}^+ \) concentration, indicating that factors in addition to the marginal cells are involved (see below).

B. Intermediate cells.

Intermediate cells form a discontinuous layer without reaching the apical or basal limit of the stria. They are melanocyte-like cells and are thought to be derived from neural crest cells. The precise function of the intermediate cells is unclear, although they do appear have a role in EP generation. Evidence for this comes from the Viable Spotted Dominant strain mutant mouse, where the intermediate cells are absent, and the animals lack an EP (Steel et al 1987). They exhibit other defects including a lack of gap junctions between basal cells and other cells, although the marginal cells appeared normal (Carlisle et al 1990) and the levels of \( \text{Na}^+\)-\( \text{K}^+ \) ATPase expression was normal (Schulte and Steel 1994). Possible roles for the intermediate cells have been advanced by Spector and Carr
(1979). They studied the roles of catalase and alpha-hydroxyacid oxidase activity in extramitochondrial metabolism and found the major activity to be limited to the intermediate cells of the stria. They suggested that intermediate cells utilised a different mode of oxidative metabolism, using lipids as an energy source, than the mitochondrial oxidative phosphorylation of marginal cells. This would provide an alternative metabolic source during oxygen deprivation which would effectively disable the oxidative metabolism of the marginal cells. A second role, they suggested, would be as sites of detoxification of oxidative byproducts.

C. Basal cells.
Basal cells are elongated and separate the marginal, intermediate cells and capillaries from the connective tissue of the spiral ligament. Complex tight junctions of a form not found elsewhere in the organ of Corti and numerous gap junctions are present on basal cells. The tight junctions act as a barrier between the perilymph of the spiral ligament and the stria (Reale et al 1975). Gap junctions are present between adjacent basal cells; between basal cells and spiral ligament cells (Forge 1984) and between the basal cells and intermediate cells (Kikuchi et al 1995). No gap junctions are found between other cell types. The large size of the basal cell gap junctions, about 15% of the total membrane area (Carlisle et al 1990), suggests a key role for the basal cells in intercellular communication within the stria.

D. Capillaries.
The sealing by tight junctions of the stria from other cochlear compartments necessitates the presence of a dedicated blood supply, provided by the strial vasculature. The delivery
of a large oxygen supply is necessary to power the ion transport mechanisms within the stria, these processes resulting in the stria having one of the highest rates of oxidative metabolism in the body (Thalman et al 1973). The requirement for oxygen to maintain EP was shown by Johnstone and Sellick (1972), who found that during anoxia the EP rapidly declines to zero. These experiments also showed that the positive EP was not due to passive ion diffusion of Na\(^+\) or K\(^+\). The K\(^+\), and probably Na\(^+\), appears to originate not from the capillary supply but from perilymph and is circulated locally from the endolymph to perilymph and back via the stria (Konishi et al 1978, Sterkers et al 1988, Offner et al 1987).

E. Models of EP generation.

It has been suggested that the generation of EP relies on an electrogenic ion transport system located in the apical or basolateral membranes of the marginal cells (Sellick and Johnstone 1974, Offner et al 1987). The model advanced by Offner et al (1987) to account for EP generation stresses the importance of Na\(^+\). Perfusion experiments showed the necessity of Na\(^+\) to the generation of EP and concluded that the positive EP was the result of the functioning of Na\(^+\)-K\(^+\) ATPase together with passive Na\(^+\) diffusion into and K\(^+\) out of the marginal cells (Offner et al 1987). This model of marginal cells has Na\(^+\) entering basally, but encountering the Na\(^+\)-K\(^+\) ATPase which pumps out most of the Na\(^+\) as it diffuses to the luminal end of the cell, with K\(^+\) accumulating in the cell. At the luminal membrane, K\(^+\) passes into the scala media and Na\(^+\) is drawn into the cell thus producing the EP with its characteristic high K\(^+\) levels.

An alternative model is the "five compartment model" proposed by Salt et al (1987), so
named after the five separate compartments they identified as a K⁺ selective electrode was advanced through the stria. This model does not necessitate the presence of an electrogenic pump in the marginal cells. Rather this model relies on the apical membranes of basal cells having a high K⁺ permeability, with passive efflux of K⁺ into the intrastrial spaces creating a positive extracellular potential within the stria. The marginal cells would then utilise the Na⁺-K⁺ ATPase present on their basolateral membranes to elevate their intracellular K⁺ level. A K⁺ permeability at the apical membrane would then allow passive diffusion of K⁺ from the cell to the endolymph.

If generation of EP relied on an electrogenic pump sited on the basolateral membrane, then a positive resting potential of the marginal cells would be required. Melichar and Syka (1987) found the marginal cell resting potential to be about 10 mv higher than the EP value, a similar finding to that of Offner et al (1987), although Salt et al (1987) found the resting potential to be the same or just greater than the EP. The localisation of Na⁺-K⁺ ATPase at the basolateral membrane of marginal cells (Kerr et al 1982) would also tend to support an electrogenic pump model.

However, there is evidence that the relationship between the maintenance of EP and the K⁺ concentration of endolymph is not a simple one. Administration of ouabain reduces the EP by a decrease in the K⁺ content of endolymph through inhibition of the Na⁺-K⁺ ATPase transport mechanism of marginal cells (Konishi and Mendelson 1970). Reductions in EP have also been observed following administration of the diuretic drugs ethacrynic acid and furosemide (Bosher et al 1973, Bosher 1980, Sterkers et al 1988). However, the mechanisms by which ouabain and the diuretics cause the EP decrease
appear to be different. Comparisons of the EP changes and $K^+$ concentration were found to differ, the reduction in $K^+$ produced by ouabain was less than furosemide although the reductions in EP were found to be almost equal (Shugyo et al 1990). These results suggested that furosemide altered EP by acting on a mechanism within the stria other than suppression of active $K^+$ transport. It is known that morphological changes occur within the stria during the period of EP depression caused by diuretics. Furosemide administration causes shrinkage of intermediate cells (Pike and Bosher 1980) whilst ethacrynic acid produces changes in basal cell gap junctional morphology (Forge 1984). Other indications that basal cell gap junctional integrity and the presence of intermediate cells are factors in EP maintenance come from studies of the Viable Dominant Spotting mouse mutant, which lacks an EP. Morphological studies have shown that these animals also lack intermediate cells and gap junctions between basal cells and other cell types (Carlisle et al 1990, Steel and Barkway 1989).

Current evidence therefore suggests that the marginal cells are not solely responsible for the generation and maintenance of the EP. Given the similarities between marginal cells and vestibular dark cells and the lack of a large vestibular potential, this suggests that the intermediate and basal cells are necessary for the EP maintenance. The specialisations on the basal cell membranes could be particularly important since they provide sealing to give the electrical isolation necessary to generate a potential difference and possess gap junctions which would allow $K^+$ a bypass route of entry into the stria from the spiral ligament. In this thesis the acquisition of the tight and gap junctional complexes on basal cells, as well as features of the maturation of the marginal cells will be studied in relation to the onset and maturation of EP order to test this hypothesis.
1.10 Embryology and maturation of the organ of Corti.

A. Formation of the otocyst.

The development of the rat otocyst has been described by Marovitz et al. (1977), the sequence of maturation appearing common to all mammals. The inner ear derives from the otic placode, a patch of thickened ectoderm on either side of the head adjacent to the rhombencephalon of the embryo. This differentiates from the surface ectoderm when cells forming the placode change morphology from loosely arranged squamous cells to densely packed columnar cells, the elongated placodal cells being twice as tall as surrounding nonspecialised epithelium. Placodal formation depends on interactions of the presumptive otic ectoderm with adjacent tissues, in particular the rhombencephalon, but also mesenchyme and neural crest cells which migrate close to the placode as it thickens. After thickening the otic epithelium invaginates to form the otic cup. Cells which will form the cochleovestibular ganglion separate from the epithelium and collect at the ventromedial aspect of the otic cup. Almost all the cochlear neurons are derived from the otic placode.

The rim of the cup fuses to form the otocyst, which sinks below the surface epithelium. The otocyst is a hollow sac of undifferentiated cells which will ultimately form the labyrinth and it is initially devoid of innervation, the nerves subsequently growing from the ganglion cells to invade the otocyst.

B. Morphogenesis of the cochlea.

During the period of the otocyst, intense mitotic activity and rapid growth occurs. During this time morphologic differentiation does not occur (Ruben 1967) although specific
regions of the otocyst appear "fated" as to future structure.

Differential growth of the otocyst initially forms a dorsal vestibular region and ventral cochlear region. The utricle and saccule arise between these two. At the same time as the vestibular portion produces the early signs of the semicircular ducts, the cochlea portion begins to elongate and coil. The cochlea develops as a ventral invagination of thick columnar epithelia. Cells at the apex undergo terminal mitosis before the base, suggesting the cochlea lengthens by adding cells at the saccular end (Ruben 1967). Note that this apical to base gradient is at odds with the base to apical maturational gradients of the cochlea and organ of Corti found at later developmental stages. The lumen of the coils becomes the cochlear duct and the medial wall differentiates to become the organ of Corti.

The sensory structures of the cochlear duct are derived from placodal ectoderm. The thickening of the posterolateral wall of the developing cochlear duct begins to differentiate at the same time as regions adjoining the duct form the scala tympani and scala vestibuli, these forcing the central scala media into its triangular shape. Fusion of the anterior wall of the scala media with the scala vestibuli forms Reissners membrane, fusion of the posterior wall of the scala media with scala tympani forms the basilar membrane.

Once the membranous labyrinth acquires all its major parts the mesenchyme condenses to form the bony labyrinth. There is evidence that the developing otocyst has an influence on the induction and morphogenesis of its capsule, i.e. chondrogenesis depends on the
presence of a normal otocyst (McPhee and van de Water 1986). The same factors which underlie normal development of neuroepithelial structures can also affect the capsule and its associated structures, the bony labyrinth and stapes footplate. The otic capsule itself has an unusual pattern of ossification. It comprises three layers, the outer periosteal layer having a normal structure resembling the long bones, a middle section retaining the partially calcified original cartilaginous capsule and an inner periosteal layer which has replaced the original capsule cartilage.

Early studies of amphibian development, reviewed by Van de Water and Ruben (1976), showed that the otic placode preceded the development of the acoustic ganglion whilst early explantation studies showed the amphibia acoustic ganglion has its origins in the otic ectoderm. Batten (1958) concluded that the statoacoustic ganglion in mammals derived from the rostral wall of the otocyst and not neural crest. The cochleovestibular ganglion derives from an aggregation of cells medial and rostral to the otocyst, the neurons arising primarily from the medial wall of the otocyst which will eventually form the macula utriculus. Embryonic ganglion cells are bipolar, their peripheral processes grow toward the receptor epithelium whilst centrally directed axons grow toward the brain (Whitehead and Morest 1985a). Elementary tonotopicity is also apparent in ganglion cell outgrowth, those dorsally located send fibres basally in the receptor epithelium, those ventrally send fibres apically (Whitehead and Morest 1985a).

Invasion of the receptor epithelium by ganglion cells occurs through the basal lamina. The basal lamina appears to form a barrier with some fibres entering as they contact the lamina whilst others travel some way along it before entering (Whitehead and Morest
Gaps in the basal lamina only appear as the fibres enter (Carney and Silver 1983). Within the epithelium, the fibres travel in a system of channels as they spread through the immature organ of Corti (Whitehead and Morest 1985b), possibly guided by factors released by the epithelial cells. Within the undifferentiated cochlear epithelia, the fibres branch in a radial direction and are directed to the surface of the epithelium (Whitehead and Morest 1985a). During this period of growth hair cells first become recognisable, thus suggesting a complex pattern of induction and trophic factor interactions.

C. The receptor epithelium: The organ of Corti.

As noted above, during the development of the inner ear, the pattern of innervation slightly precedes the pattern of sensory receptor differentiation (Sher 1971). This section describes the sequence of maturation of the organ of Corti following the appearance of recognisable hair cells. The sequence of events is common to all mammals, the period over which the events occur however is highly species dependent.

In several mammalian species, the morphological and functional maturation of the cochlea occurs postnatally. This has been studied in several species of altricial rodents, most extensively in the rat (Carlier et al 1979, Lenoir et al 1987, Roth and Bruns 1992a,b) but also in mice (Kikuchi and Hilding 1965, Kraus and Aulbach-Kraus 1981, Lim and Anniko 1985), hamsters (Kaltenbach and Falzarano 1994) and gerbil (Finck et al 1972). At birth in these species the undifferentiated sensory epithelium, the organ of Corti, is composed of a mass of columnar cells. Extensive changes take place over a short period typically lasting 3 - 4 days, this maturation having been described as proceeding with a
base to apex gradient along the cochlear spiral (Rubel 1978 for review).

The immature organ of Corti itself is associated with several features, including lack of spaces, a thickened basilar membrane, large numbers of microvilli on the apices of cells facing the scala media. Each sensory and supporting cell has a prominent kinocilium, which is a true cilium. The kinocilium of the hair cells may assist in morphologically polarising the apical surface of the cells. The organ of Corti itself has the appearance of a thickened epithelial layer and at this stage is referred to as Kollikers’ organ. The tectorial membrane forms from an extracellular matrix which lies above the luminal surface in an amorphous layer and contains a large amount of collagen II, the cells of the epithelial ridge cells appearing to secrete the matrix (Lim and Anniko 1985). During development, the tectorial membrane is attached at the level of the outer row of Dieter’s cells by marginal pillars, this anchorage breaking just prior to the onset of functional hearing. Upon formation of the tectorial membrane, the cells of greater epithelial ridge begin to lose their apical microvilli and regress to form the inner spiral sulcus.

The IHCs arise from the greater epithelial ridge and the OHC from the lesser epithelial ridge of Kollikers’ organ. The early stereocilia emerge from the surrounding microvilli, which cover the apical surfaces of both the sensory cells and supporting cells. A kinocilium is also present on all cells, which has disappeared usually by the time of the onset of functional cochlear responses. The IHC are separated from the first row of OHC by tall columnar cells which differentiate into inner and outer pillar cells, some time after the hair cells appear. As the inner and outer pillar differentiate, the tunnel of Corti opens creating a fluid filled arch. Spaces also begin to appear around the OHCs, the spaces of
Nuel, whilst the processes of the Deiters' cells become thinner. The supernumary microvilli are gradually lost from the apical surfaces of the hair cells and sensory cells.

In the gerbil, the subject of this study, the maturation of the organ of Corti takes place over the first three weeks following birth. Aspects of the postnatal development of the gerbil organ of Corti have been addressed in a number of studies: the maturation of cochlear potentials (Finck et al 1972, Woolf and Ryan 1984, Harris and Dallos 1985, Yancey and Dallos 1985, Woolf et al 1986, Harris et al 1990, Arjmand et al 1988, Echteler 1989, McGuirt et al 1995); changes in the afferent nerve population of the organ of Corti (Echteler 1992); the maturation of middle ear structures (Woolf and Ryan 1988, Cohen et al 1992); otoacoustic emission development (Norton et al 1991) and changes in the tonotopic map of the higher auditory centres (Sanes and Rubel 1988, Sanes et al 1989). The development of motility in isolated outer hair cells (OHC) of gerbils has also been examined by He et al (1994). The above studies have tended to focus on the maturation of a few specific features, whilst one of the purposes of this thesis is to provide baseline data regarding the structural and physiological maturation and the temporal relationship between the two.

Parallel studies of the onset of physiological responses and morphological features have often attempted to define the appearance of a specific morphological event which is the crucial factor in the onset of function. These have included synaptic formation (afferent or efferent), the opening of spaces, the freeing of the tectorial membrane, stria vascularis and onset of EP, as well as structurally related changes which would allow the shearing motion of the basilar membrane- tectorial membrane to occur. It would, however, appear
unrealistic to expect a single event to be the determining factor for the onset of auditory function. It appears, rather, that changes occur in a quite rigid sequential pattern, with some events being dependent on those preceding it whilst others appear independent of preceding changes. One of the aims of this thesis is to examine the relationship between several aspects of maturation during the postnatal development of the organ of Corti in gerbils. In particular, the relationship between sensory cell, supporting cell and basilar membrane maturation and the onset and maturation of functional cochlear responses which represent both electrophysiologic and active mechanical properties of the organ of Corti will be studied. The maturation of the stria vascularis and the onset and growth of EP will also be examined, with particular emphasis on the formation of junctional complexes within the stria and their possible roles in generation and maintenance of EP.
FIGURE 1.1.

Schematic cross section of the elements of the mammalian auditory organ.

FIGURE 1.2.

Schematic cross section of the mammalian cochlea. b = bony capsule; c = organ of Corti; m = scala media; v = scala vestibuli; t = scala tympani; mo = modiolus; n = auditory nerve.
FIGURE 1.3.

Schematic cross section of the organ of Corti (From Kim 1986).
FIGURE 1.4.

Schematic diagrams of IHC and OHCs. Left: IHC. s = stereocilia; t = tip-links; c = cuticular plate; m = mitochondria; l = lateral membrane; n = nucleus; a = afferent nerve; v = vesicles. Right: OHC. p = cytoskeletal pillars; lc = lateral cisternae; j = junctional region with supporting cell; e = efferent nerve.
FIGURE 1.5.

Innervation patterns of the mammalian cochlea. SO = superior olivary complex of brainstem; L = lateral superior olive; M = medial superior olive; CN = cochlear nucleus; TI = type I afferents; TII = type II afferents; OC = organ of Corti; i = inner hair cell; o = outer hair cell.
FIGURE 1.6.

Schematic cross section of the stria vascularis. SM = scala media; MC = marginal cell; C = capillary; IC = intermediate cell; m = mitochondria; BC = basal cell; t = tight junction; g = gap junction.
CHAPTER 2.

METHODS

This chapter describes the methods employed in the structural and functional studies of the maturation of the organ of Corti presented in this thesis. The techniques for dissection, fixation and preparation of tissues for scanning electron microscopy (SEM), thin sections for light and transmission electron microscopy (TEM) and freeze fracture are common to both the organ of Corti and stria vascularis studies. The methods for the recording of CM, CAP and stimulus frequency otoacoustic emissions (SFOAE) are also detailed. Additional methods for the immunofluorescent labelling of the tight junctional protein ZO-1 in the organ of Corti, for endocochlear potential recording from the scala media; Na\(^+\)-K\(^+\) ATPase reactivity and quantitative analysis of junctional maturation in the stria vascularis are described separately at the beginning of the relevant results sections.

2.1. Establishment and maintenance of the gerbil breeding colony.

Twelve male and twelve female gerbils (*Meriones unguiculatus*) were obtained from a specialist supplier and paired to form a colony of 12 breeding pairs. The breeding performance of each pair was closely monitored, with pregnant animals being checked twice daily, the day of birth of pups being defined as day 0. Pups which were not taken as pre-weaners for experimental purposes were sorted by sex with littermates only being held in the same cages. This allowed the subsequent pairing of unrelated animals to take place and the establishment of further breeding pairs. This was necessary to prevent inbreeding occurring within the colony and to minimise the appearance of any genetically
related defects.

2.2. Preparation of specimens for structural studies.

Young gerbils from the breeding colony were taken at intervals of two days after birth (DAB) between 2 DAB to 20 DAB inclusive. Adult tissue was obtained from animals at least 3 months old. A total of 193 animals were used in these studies.

2.3. Dissection and fixation.

Animals were killed with an overdose of pentobarbitol, decapitated and the bullae quickly removed. The bullae were widely opened to gain access to the cochleae and holes made in the apex and lateral wall. Fixative was then perfused through the round window and allowed to exit through the hole made at the apex. The fixative was 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3, total osmolarity 550 mOsm). The specimens were immersed in fixative for 1.5 hours under gentle rotation at room temperature. The cochleae were then dissected under 0.1 M Cacodylate buffer, the organs of Corti and stria vascularis being removed for further processing.

2.4. Processing for microscopy and freeze fracture.

A. TEM and light microscopy.

For light microscopy and TEM, the cochleae were fixed as above. The whole organ of Corti was dissected out with the modiolus intact from animals over 8 DAB and post fixed in 1% cacodylate buffered osmium tetroxide (OsO₄) for 1 hour at room temperature. For cochleae from animals less than 8 DAB, which is prior to ossification of the cochlear wall, the cochlear capsule was removed and the whole cochlea processed as a single piece.
of tissue. Samples were then washed five times in buffer for 5 minutes each, this being followed by an alcohol series to 70% ethanol and en bloc staining for 1 hour in 1% uranyl acetate in 70% ethanol solution. The tissue was then taken through a further dehydrating alcohol series to 100% ethanol. The tissue was immersed in a 3:1 propylene oxide: resin solution under rotation for 2 hours, followed by 50% resin in propylene oxide overnight, then embedded in 100% resin and cured in an oven at 60° C. for 24 hours. The blocks were cut in 1 μm sections and stained with toluidine blue for light microscopy, then sectioned at 80-100 nm and stained using uranyl acetate and lead citrate prior to mounting on grids for TEM examination.

B. Scanning electron microscopy (SEM).

Samples of organ of Corti tissue were prepared for SEM using the TOTO process described by Davies and Forge (1987). This method involves the use of thiocarbohydrazide (TCH) as a mordant for a conductive coat of OsO₄. Three layers of osmium are built up, the second and third layers being attached to a layer of TCH.

For SEM, the wall of the cochlear capsule was removed, the tissue dissected out under buffer at room temperature and samples post fixed in OsO₄ as described above. Following 5 x 5 minute washes in distilled water, the samples were immersed in a freshly prepared, filtered solution of 0.5% TCH for 20 minutes. The samples were washed again 5 times for 5 minutes each in distilled water before being returned to OsO₄ for 1 hour. The sequence of wash-TCH-wash-OsO₄-wash was then repeated. The samples were then dehydrated through an ethanol series to 100% ethanol, culminating in 3 changes of 100% ethanol. The samples were critical point dried from liquid CO₂. The tissue was then mounted on aluminium stubs and sputter coated.
C. Freeze fracture.

Following fixation in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, the organ of Corti was separated into segments from the apical, middle and basal turns. The samples were infiltrated with 25% (v/v) glycerol (1:1:2 glycerol: distilled water: 0.2 M sodium cacodylate buffer) under gentle rotation for 45 minutes. Small sections of tissue were placed into the holes of a gold specimen holder using a yeast/glycerol paste as a mountant, a second gold holder was then placed carefully over the specimen, which was held in place by the yeast/glycerol paste. The tissue sample "sandwich" was rapidly frozen in a constantly stirred 4:1 propane: isopentane mixture cooled by liquid nitrogen. If not immediately required the samples were stored in liquid nitrogen. Samples were fractured in a Balzers BAF400 apparatus at 168 K and the exposed surfaces unidirectionally shadowed with platinum-carbon. The replicas were floated onto distilled water and transferred to 40% chrome-sulphuric acid to dissolve the underlying tissue. The replicas were then washed with distilled water before mounting on grids for TEM.

2.5. Quantitative data: The organ of Corti.

Quantitative data from the maturing organ of Corti was obtained from calibrated light and TEM micrographs using a VIDS V image analysis system (Synoptics). The measurements were made from the regions as shown in the schematic Figure 2.1. Analyses of junctional morphology OHCs and IHCs, intramembrane particle density of the lateral walls of OHCs and IHCs were made from calibrated TEM prints of freeze fracture replicas.

Measurements of the apical junctional region of OHCs and IHCs were made from prints of calibrated magnification 40k. The total apical junctional depth was measured by
drawing lines perpendicular to the cells' apical surface and taking the average length of 4 measurements for each cell. The measure of the complexity of the junctions of OHCs was based on the definition of Wolburg et al (1994), who defined a complexity index as the density of branch points on the tight junctional strands. Due to the continuous nature of the junctional strands in the gerbil making measurements of the length of individual strands impractical, the complexity index was defined as the number of branching points per unit area. The more complex nature of IHC junctions precluded a similar analysis since several strands from the tight junctional regional descend into the basalmost region creating "whorls" of strands (see Figure 3.26). The number of junctional strands was determined by counting the number of strands which intersected random lines drawn across the junctional region from the apex to the base of the junction. Measurements were made from five OHCs from each of four animals in each age group. For each OHC the mean of 4 measurements was obtained. A smaller sample of IHCs was used, two IHCs from each of two animals in each age group.

The density of the intramembrane particles (IMPs) on the lateral membrane of OHCs was calculated by counting the number of particles in an area so drawn as to exclude any large discontinuities, from prints of calibrated magnification of 125k. Counts were made from six cells in each of six animals from each age group. Analysis of the spacing between the subplasmallemal pillars of OHCs was made from TEM prints of thin sections, the prints having a calibrated magnification of 125k. Standard errors for all measurements were calculated.

2.6. Physiological response measurements: CM, CAP and SFOAEs.
Experiments involving the recording of stimulus-evoked potentials were carried out with the animal in a sound-proofed booth.

A. Anaesthesia.

The anaesthetic regime employed in these studies was selected in consultation with the Home Office veterinary inspector. Animals free from middle ear infection were selected from the colony.

Gerbils from which only SFOAEs were to be recorded were anaesthetised with Hypnorm (Janssen, 0.25ml/kg intra-muscular) and Diazepam (25mg/kg intra-peritoneally). Heart and respiration rates were monitored using a Hewlett Packard Patient Monitor (HP78354A) with a minimum heart rate set at 250 beats per minute and respiration rate minimum of 80 per minute for alarm activation. Core temperature was monitored via a rectal probe and maintained at 39°C. with a heating blanket. Anaesthesia was maintained at a level which allowed insertion of the acoustic stimulus delivery probe into the animals' meatus without discomfort or attempts being made to dislodge it. Supplementary doses of the appropriate drug were administered as required.

Animals which were to undergo invasive surgical procedures were anaesthetised using Diazepam (5mg /kg intra-peritoneally) and Hypnorm (1ml/ kg intra-peritoneally). Body temperature was maintained by wrapping the animal in cotton wool and use of the heating blanket. Heart and respiration rates were monitored as above. Supplemental doses of anaesthetic were given as required to maintain surgical anaesthesia.

B. Surgical procedures.
Once a surgical level of anaesthesia was attained, denoted by the absence of the paw-pincher reflex, the animal was secured in a head holder. In animals over 14 DAB a tracheal cannula was inserted. The pinna, skin and musculature covering the inferior-posterior and tympanic chambers of the bulla were removed and a hole made through the bone to give access to the round window. Any fluid in the chamber was removed using an absorbent wick. In animals less than 16 DAB, fluid and mesenchymal tissue frequently filled the bulla and middle ear cavities. This was carefully removed using a combination of absorbent wick and gentle suction through a small 1 ml syringe fitted with a 1 mm bore PTFE tube.

C. Measurement of cochlear microphonic and compound action potentials.

A recording electrode was constructed from teflon coated silver wire (Clark Electromedical). The final 1 mm of insulation was stripped from the wire prior to placement. The electrode was placed manually, aided by an operating microscope, onto the ridge of the round window and secured with cyanoacrylate adhesive. The reference electrode was placed below the skin on the animals' neck and the earth was secured in the forelimb musculature.

The signals recorded at the round window were passed through a Neurolog NL104 A.C. preamplifier and band limited by a Neurolog NL125 filter unit, high pass 500 Hz. The filtered waveform was displayed on a two channel digital storage oscilloscope (Gould 1425). Measurements of CM and CAP amplitude were taken from stored waveforms, which were then printed out on a Gould 6120 plotter.
D. Measurement of stimulus frequency otoacoustic emissions.

The method used to obtain continuous-tone-evoked OAEs was that originally described in Kemp, Brass and Souter (1990), with modifications described in Brass and Kemp (1991, 1993). Briefly, a probe consisting of two miniature loudspeakers (Knowles BP1712) and one microphone (Knowles EA1843) was inserted into the meatus of the subject, the probe was connected to a signal analysis interface capable of generating the acoustic stimuli and measuring the meatal sound field under computer control. The probe assembly and the stimulus generation and recording system were calibrated prior to use.

One loudspeaker produced a constant tone, the stimulus (F2), at a preselected frequency, which evokes a SFOAE. Tone bursts, the suppressor (F1), with 1 ms rise times were produced by the other loudspeaker. This tone suppresses the SFOAE produced by the stimulus tone. Figure 2.2 shows schematically how the stimuli are presented and how the residuals are produced. The stimulus tone in sections B and D is inverted relative to that in sections A and C to produce a continuous waveform. The tone bursts were presented with the same polarity in sections C and D.

The sound field in the meatus was recorded by the microphone and digitised at 40 μs intervals via a 12 bit analog to digital converter (ADC). The ADC output was manipulated and "subaveraged". This involved summing the four sections A to D which produced a waveform the length of one section. This averaging process cancelled that part of the sound field linearly related to the stimulus and suppressor tones to reveal a nonlinear residual response. The sound stimuli were presented repeatedly and the residuals themselves averaged to reduce noise contamination. Typically 512 averages
were performed. Two separately averaged residuals were collected by adding the subaveraged data alternately to one of two averaging buffers. This allowed confirmation of the presence of a repeatable residual due to an OAE, rather than artifactual waveforms which may have contaminated the waveform due to small movements of the probe, for example. Filtering of the residual waveforms, if required, was carried out post averaging by zero time shift digital filters. The sound pressure levels of the two stimuli in the meatus were monitored via the output of a secondary ADC which was displayed unaveraged.

The above processes, together with fast fourier transform (FFT) spectral analysis, comparison of the two collected residuals and filtering of the waveforms, were performed digitally by a custom written computer programme in real time. The noise floor of the system was typically -25 dB SPL.

The residual produced by the above manipulations is a measure of the degree of suppression of the SFOAE produced by the tone burst, the suppressor tone in Figure 2.2, or more correctly the difference, either in phase or amplitude, between the OAE generated by the stimulus in the presence and absence of the suppressing tone.

E. Acoustic stimulus generation and delivery.

i) Selection of Stimulus Frequency.

Frequencies of 1.5 kHz and 4 kHz were chosen; 1.5 kHz to represent a low frequency stimulus and 4 kHz because it represents the most sensitive frequency region in the adult gerbil cochlea. Previous work on human and guinea pig subjects (Brass and Kemp 1993,
Souter 1995a) indicated that suppressor tones just higher in frequency than the stimulus tone were effective in producing SFOAE residuals, and accordingly stimulus tones of 1.5 kHz and 4 kHz were paired with suppressor tones of 1.8 kHz and 4.8 kHz respectively.

ii) *Acoustic stimulus presentation.*

The acoustic stimuli to generate the CM were presented as continuous tones and the CAP was evoked using tone bursts with 1 ms rise times. These waveforms were computer generated sinusoids (windowed in the case of the tone bursts) at the selected frequencies (1.5 and 4 kHz) and delivered through one of the loudspeakers housed in the meatal probe assembly. The sound field in the meatus was monitored by the miniature microphone. Due to the possibility of distortion being produced by the loudspeaker and the probe assembly, the maximal output of the system was limited to 75 dB SPL.

The collection of data was usually limited by time constraints due to contamination of the waveforms caused by respiratory noises and probe slippage which necessitated repeated stimulus presentations to obtain artifact free data.
FIGURE 2.1.

Schematic cross-section of the middle turn of the gerbil organ of Corti at 10 DAB. O, outer hair cells; I, inner hair cell; D, Deiters' cells; IP, inner pillar cell; OP, outer pillar cell; TC, tunnel of Corti; SN, space of Nuel; SV, spiral vessel; ZA, zona arcuata; ZP, zona pectinata. Measurements referred to in the Results were obtained from the positions illustrated. OCW, organ of Corti width; D1 and D2, width of the heads of the first and second row Deiters cells; PH, width of the pillar cell head; BMT, total basilar membrane thickness; HM, hyaline mass thickness; TCL, epithelial tympanic cover layer; IPL, inner pillar length; OPL, outer pillar cell length; PCS, pillar cell separation. The angles formed by the outer and inner pillar cells and the basilar membrane were also measured as shown.
FIGURE 2.2.
Schematic representation of the arrangement of the stimulus tones and the manipulations of the waveform blocks carried out to produce the residual. The acoustic stimulus is presented in four consecutive time periods, A, B, C and D, each of 40 ms duration. The continuous stimulus tone F2 was presented in periods A and C, and inverted in periods B and D, in such a way as to produce a continuous waveform (panel I). The suppressor tone F1 was presented in two bursts, cosine windowed with a 1 ms rise time and of the same polarity, in periods C and D (panel II). The resultant sound field is shown in panel III. The results of the subtractions of the time periods (A-B) and (C-D), followed by the final calculation of (A-B) - (C-D) which produces the residual are shown in panel IV. (From Souter 1995a).
Section A  Section B  Section C  Section D

Stimulus tone  \( F_2 \)
Suppressor tone  \( F_1 \)
Sound field in meatus

\[ (A-B) - (C-D) = A-B-C+D \]
CHAPTER 3.

RESULTS.

3.1. PHYSIOLOGICAL RESPONSE MATURATION.

3.1.1. CM and CAP.

Figure 3.1 shows the maturation of the CM between 12 and 20 DAB. Small CM potentials to 4 kHz stimulus tones at 75 dB SPL were just recordable at 12 DAB and showed a rapid rise between 12-14 DAB, increasing at about 30 μV per 24 hours. By 16 DAB the CM response had attained about 75% of the mature response. The maximum CM continued to increase between 16-20 DAB, the rate of increase being about 100 μV per 24 hours, with the CM level recorded at 20 DAB being 90% of the adult value. The 1.5 kHz CM was measurable with stimuli at 75 dB SPL at 12 DAB, being slightly greater than that obtained with the 4 kHz tone. The 1.5 kHz CM showed a slightly greater increase in level between 12-14 DAB, 40 μV per 24 hours, than that of the 4 kHz CM, and reached 60% of the adult DAB value by 20 DAB.

The growth of the CAP to 1.5 and 4 kHz tone bursts at 75 dB SPL is shown in Figure 3.2. The CAP was not measurable until 14 DAB, and grew less rapidly than the CM over the period 14-20 DAB, when it had attained 85% of the mature response magnitude for the 1.5 kHz CAP and 93% of the response obtained from adult DAB subjects for the 4 kHz stimulus. After 16 DAB, the maximum response to the 1.5 kHz tone burst became larger than that obtained with the 4 kHz tone burst.

Summary.

The CM was the first acoustic stimulus evoked potential to be recordable, the CAP onset
lagging CM onset by approximately 48 hours. There was no difference between the stimulus frequencies for CAP growth, however the low frequency elicited CM was recordable before the higher frequency CM, the latter exhibiting a more rapid growth rate between 12-18 DAB. The CM and CAP both continued to increase from 20 DAB onward, with the lower frequency stimulus producing larger potentials.

3.1.2 SFOAEs.

SFOAEs at 1.5 kHz and 4 kHz were first measurable at 14 DAB with stimulus levels of 75 dB SPL. Figure 3.3 shows the 1.5 kHz SFOAE residual recorded in the meatus of a 20 DAB gerbil, with filtered and unfiltered waveforms. The latency of the residual with respect to the stimulus (Figure 3.3 upper panel) is clearly visible, indicating an intracochlear origin for the signal. The growth of the SFOAE residual levels during maturation is shown in Figure 3.4. The level of the 1.5 kHz SFOAE residual at 14 DAB was just measurable at -15 dB SPL, the 4 kHz SFOAE residual larger at -9 dB SPL. The SFOAE residuals grew rapidly in amplitude between 14-20 DAB, by which time the 1.5 kHz residual was 80% of the value obtained from adult DAB subjects and the 4 kHz SFOAE 90% of 32 DAB value. The 4 kHz and 1.5 kHz SFOAE residual grew at a mean rate of 5 dB per 24 hours between 12 and 20 DAB. By the 20 DAB the SFOAE had not reached the adult values, both 1.5 and 4 Khz SFOAEs increased by about 5 dB SPL between 20 DAB and the adult values.

Summary.

The onset of recordable SFOAEs occurred after the CM onset but at the same time as the CAP onset. In contrast to CM and CAP recordings, the higher frequency SFOAE at 4 kHz was larger throughout the time period studied. The SFOAE amplitude continued to grow after 20 DAB. This, together with the finding of continuing evoked-potential
increases during this time, suggests both mechanical and electrophysiologic factors continue to mature after 20 DAB.

3.2. STRUCTURAL MATURATION.

The principal morphological findings described in this section of the thesis are the demonstration that maturation of the accessory structures, i.e. the pillar cells, basilar membrane, the opening of the intra organ of Corti spaces and the lumen of the inner spiral sulcus, occurs initially in the middle turn, spreading to the base and finally the apical turn. The time course of maturation of the pillar cells and basilar membrane encompasses the period of onset and maturation of cochlear responses, and is not complete until 20 DAB.

3.2.1 The organ of Corti.

A. Sensory cell maturation: SEM views.

SEM views of the apical surface of the organ of Corti between 2- 20 DAB are shown in Figure 3.5. At 2 DAB two hair cell populations, the inner hair cells (IHC) and OHCs, were identifiable in all three turns (Figure 3.5 A- C). Each hair cell bore well defined stereociliary bundles. Kinocilia were present, situated on the outer side of the stereociliary bundle, as were large numbers of microvilli which covered the inner apical surface of the IHCs and OHCs. Each microvillus was joined to its neighbours by six cross-linking fibres. The apical surfaces of the supporting cells, each with a kinocilium, were also covered by profuse microvilli.

By 6 DAB (Figure 3.5 D- F) the distance between the outer two rows of OHCs had begun to increase in the middle turn (Figure 3.5 E); this was more obvious by 8 DAB.
Figure 3.5 G-I). 8 DAB samples also showed a gradation in the density of microvilli on the apical surface of OHCs from base to apex. Microvilli were still present in large numbers on OHCs from apical and middle turns and on IHCs and supporting cells in all turns. However, basal turn OHCs had lost almost all of their extra microvilli (Figure 3.5 I). The OHCs from the middle and basal turns at 10 DAB had lost their apical surface microvilli; however they remained on all IHCs and apical turn OHCs (Figure 3.5 J). By 12 DAB, the microvilli had been lost from the apical surfaces of all OHCs, excepting those in the extreme apex, which retained an immature appearance (Figure 3.5 K). At 16 DAB, the surface of the reticular lamina in all turns had taken on an adult-like appearance (Figure 3.5 L-N).

Summary.

Immature hair cells are identifiable by a prominent kinocilium, profuse microvilli and rows of stereocilia, features present on hair cells from all turns at 2 DAB. Both IHCs and OHCs mature with a base to apex gradient. Taking the presence of microvilli on the apical surfaces as an indicator of cell maturity, the OHCs appear to mature before IHCs, the IHCs lagging the OHCs by about 48 hours. Basal turn OHCs are the first hair cells to lose their microvilli.

B. Supporting cells: A middle turn outward gradient.

The width of the organ of Corti in the apical, middle and basal turns was measured from the IHC border with the pillar cell heads to the strial side of the outermost OHC row. The means of the measurements are plotted in Figure 3.6 from the data in Table 3.1. At 2 DAB, the width was found to be greatest in the apical turn (41 μm) with the basal and middle turns being similar at about 38 μm. Little increase was seen between 2 and 6 DAB but by 8 DAB the organ of Corti had become widest in the middle turn, about 50
μm, with the apical and basal turn having mean values of 46.4 μm and 42.9 μm respectively. By 10 DAB, the mean values for the organ of Corti width were 66.5 μm in the middle turn, 59.3 μm in the apical turn and 51.9 μm in the basal turn. Increases in width continued until 20 DAB, when almost adult values were reached, with the middle turn remaining widest at 84 μm, the basal turn 65 μm and the apical turn 63 μm. This increase in width derived mainly from two sources; increases in the sizes of the heads of the inner pillar cell (IP) cells, and increases in the heads of the Deiters’ cells (Figure 3.7). The increase in the IP heads in the middle turn began between 8 and 10 DAB, and is described in detail below, whilst the increase in the width seen up to 8 DAB was accounted for almost exclusively by an increase in the size of the second row of Deiters’ cells. These increase from about 3.5 μm at 2 DAB in the middle turn, with similar values in the apical and basal turns, to about 14 μm in the middle turn, 6 μm in the apical turn and 4 μm in the basal turn by 8 DAB (Table 3.1). Increases in the organ of Corti width between 12 and 16 DAB were mainly due to pillar cell head enlargement, the Deiters’ cell heads increasing by about 5 μm in the period 12- 20 DAB whilst the pillar heads increased by about 15 μm in the middle turn over the same period (Figure 3.7).

**Summary.**

Maturation of the supporting cells, as shown by increases in cell apical head width, proceeded from the middle to basal and finally apical turns. The heads of the first row of Deiters’ cells were the first to enlarge from 6 DAB onward, with increases in the IP head width not commencing until about 8 DAB. The increase in the width of the organ of Corti from 2- 8 DAB was the result of Deiters’ cell head enlargement and from 10 DAB onward as a consequence of IP head enlargement.
C. The body of the organ of Corti.

Figure 3.8 A-C shows light micrograph sections of the maturing organ of Corti from 2-8 DAB. At 2 DAB the hair cells and supporting cells were in close contact, forming a compact cell mass on the basilar membrane with no tunnel of Corti or spaces of Nuel present (Figure 3.8 A). Although the lumen of the inner spiral sulcus had begun to open at 4 DAB (Figure 3.8 B), by 8 DAB there was still no evidence of any substantial space opening in the region of the sensory cells (Figure 3.8 C). By 10 DAB, the tunnel of Corti had appeared, Figure 3.9 A-C show light micrograph sections of the apical, middle and basal turns of the same cochlea at 10 DAB. No spaces are evident in the apical turn (Figure 3.9 A). However, the tunnel of Corti and spaces of Nuel are well developed in the middle turn (Figure 3.9 B). The basal turn shows a lesser degree of space opening, with the spaces around the OHCs being particularly undeveloped (Figure 3.9 C). Between 12 (Figure 3.10 A) and 14 DAB the middle and basal turns exhibited a similar degree of space opening. However, the apical turn continued to lag in maturational state. By 20 DAB, the intra organ of Corti spaces had opened to their mature dimensions (Figure 3.10 B).

Summary.

The intra-organ of Corti spaces were first evident at 10 DAB. The tunnel of Corti first appeared as a substantial opening in the middle turn at 10 DAB, with a smaller space in the basal turn but no spaces evident in the apical turn. The spaces of Nuel also began to appear around the innermost row of OHCs at 10 DAB. By 14 DAB the middle and basal turn had openings of similar size, whilst the apical turn continued to have smaller spaces.

D. Inner spiral sulcus and tectorial membrane.

The progressive opening of the lumen of the inner spiral sulcus between 2-20 DAB can
be seen in Figures 3.8 and 3.10. At 2 DAB, tall columnar cells filled the region which would become the lumen, their apical surfaces covered by profuse microvilli (see Figure 3.14). The lumen had begun to open by 4 DAB in the middle turn (Figure 3.8 B), was present by 6 DAB in the basal turn and by 8 DAB in the apical turn. The lumen opened first on the modiolar side, opening in the direction of the hair cell region as the cells which had filled the lumen contracted (Figure 3.8 B and C). By 16 DAB the lumen had opened almost fully in the middle and basal turns, the apical turn lagging these by several days. The precise mechanism by which the sulcus opens is not known. Throughout the period of the opening of the sulcus, there was no evidence of cell death seen within the body of the greater epithelial ridge, nor was cell debris seen in the developing sulcus.

At 2 DAB, the major part of the tectorial membrane was in close contact with the cells of the greater epithelial ridge, with the marginal region extending over the OHCs (Figure 3.11 A). The tectorial membrane was composed of thick fibrils running radially and longitudinally almost perpendicular to each other. Within this net was a mesh of thinner fibres running at angles to the thicker fibres. By 4 DAB this meshwork of fibres had increased in density, with the larger fibres increasing in diameter (Figure 3.11 B). Amorphous material appeared to contact the OHCs, with column-like trabeculae attached to the OHC stereocilia (Figure 3.12 A) at 6 DAB and by 12 DAB the tectorial membrane had taken on an almost mature appearance, with the thicker fibres almost obscuring the thinner components (Figure 3.12 B). During the period 2-12 DAB, the cells of the greater epithelial ridge remained covered in dense microvilli. Figure 3.13 A shows that these microvilli appear to be in contact or embedded in an amorphous layer of material which lies below the fibres comprising the tectorial membrane, which appear to originate
in material released into the subtectorial space by the cells of the greater epithelial ridge (Figure 3.13 B).

**Summary.**

The opening of the lumen of the inner spiral sulcus commenced in the middle turn at 4 DAB and appeared fully open in all turns by 18 DAB. The tectorial membrane appeared to condense from amorphous material secreted by the cells forming the greater epithelial ridge. Maturation proceeded with an increase in the density of fibrils forming the tectorial membrane and appeared almost mature by 12 DAB.

**E. Pillar cells.**

At 2 DAB the pillar cells and the hair cells were in close contact along their lateral walls. The pillar cell bodies were distinguished from the surrounding cells by the presence of vertically oriented microtubule bundles, which were apparent in the upper half of the cell body (Figure 3.14 A). At 8 DAB in the middle turn the bundles extended further toward the base of the pillar cell (Figure 3.14 B) and by 10 DAB changes in the orientation of the microtubules in the inner pillar cells (IP) had occurred. The apical region of the microtubule bundles began to bend so that they ran parallel to the surface of the reticular lamina, extending over the outer pillar (OP) cell (Figure 3.15 A). By 16 DAB the IP heads had continued to expand, increasing the separation between the IHC and the OHCs (Figure 3.15 B).

Figure 3.16 A shows a section through the organ of Corti from the middle turn at 6 DAB. The pillar cell bases lie above the large spiral vessel, and the microtubule bundles in the apical region of the cell body have organised into discrete bundles (Figure 3.16 B, compare with Figure 3.14 A). Adherens junctions were present between the OP and IP
lateral membranes and were evident at the neck of the OHC and IP in the region which forms the IP-OHC junctional complex in the mature organ of Corti. However there was no evidence of junctions between the supporting cell and OHC lateral walls (Figure 3.16 C). At 8 DAB, whilst the supporting cell was still closely adjacent to the OHC (Figure 3.17 A), the process of separation of the OHC and supporting cells had begun; a "clean" break appearing along the length of the lateral walls (Figure 3.17 B). Adherens junctions were still present between IP and OPs, and IP apical regions and adjacent OHCs but did not occur between the OPs and OHCs (Figure 3.17 C). By 10 DAB the tunnel of Corti and spaces of Nuel had opened substantially in the middle turn (Figure 3.18 A). The lateral walls of both IPs and Ops were covered with large numbers of microvilli (Figure 3.18 B). These persisted beyond 16 DAB but were absent by 20 DAB.

During maturation, the IP and OP cells increase their lengths and alter their orientation with respect to the basilar membrane. Quantitative data on the pillar cells is given in Table 3.1 and the change in length of IP and OP from the middle turn is shown in Figure 3.19. At 2 DAB, the IP and OP were oriented almost perpendicularly to the basilar membrane, at respective angles of about 85 and 84 degrees (Table 3.1). The pillars in the apical turn were slightly longer than those of the middle or basal turns. By 6 DAB, the angle of the IP in the middle turn had decreased to around 75 degrees, but there was little change in the orientation of the OPs. The movement of the IP bases toward the modiolus further reduced the angle to 70 degrees by 8 DAB, similar to that found in the adult. The bases of the OPs continued to move away from the modiolus, the angle decreasing from 78 degrees at 8 DAB to around 50 degrees, the adult value, by 12 DAB. As a result of the movement of the pillar cell bases, the angle between the pillars

82
increased from around 12 degrees at 2 DAB to 63 degrees in the adult. The opening of the tunnel of Corti coincided with this movement of the OP bases. The gradual shift in position of the pillar cells results in their bases resting on two separate regions of the basilar membrane, the IPs on the edges of the bony spiral lamina and the OPs at the edge of the zona arcuata (the inner region of the basilar membrane), just above the zona pectinata (the outer region of the basilar membrane) and its associated hyaline mass. The relative lengths of the pillars also changed, with the pillars of the middle turn becoming longest by 8 DAB (Table 3.1). The inner pillars were longer than the OPs from 2 DAB onward, by 10 DAB the pillars of the basal turn becoming longer than those of the apical turn. This increase in length was accompanied by an increase in the size of the heads of the IPs, which moved over the heads of the OPs, replacing them in views of the reticular lamina by 6 DAB. There was little change in the size of the pillar heads between 2 and 6 DAB, being in the range 2.75 to 3.8 \( \mu \text{m} \) during this period with little variation between the turns. Between 8 and 10 DAB, the pillar heads increased dramatically, particularly in the middle turn, attaining lengths of around 13 \( \mu \text{m} \) in the middle turn as opposed to 7 \( \mu \text{m} \) in apical and basal turns (see Figure 3.7). By 12 DAB this difference had become less pronounced, and by 20 DAB the pillar heads were between 25 and 28 \( \mu \text{m} \) in both the basal and middle turns.

To determine the extent of IP and OP separation, the distance between the strial edge of the OP base and the modiolar edge of the IP base was measured in the middle turn (Table 3.1). Between 2 and 8 DAB, prior to any spaces between the IP and OPs, the distance increased from 25.6 \( \mu \text{m} \) to 52 \( \mu \text{m} \), this being due to increasing cell size. By 10 DAB, as the spaces began to form in the middle turn, the separation increased to 70 \( \mu \text{m} \), further
increasing to 85 \( \mu m \) by 16 DAB and reaching an almost mature value at 20 DAB of 100 \( \mu m \).

**Summary.**

Immature pillar cells between 2 and 6 DAB were oriented almost perpendicular to the basilar membrane and contained bundles of vertically oriented microtubules. Between 8 and 10 DAB, the microtubule bundles began to change orientation in the apical region of the cells, with a coincident enlargement of the heads of IP cells. The process of opening of the tunnel of Corti commenced about 8 DAB with separation of the pillar cell bodies from the adjacent OHC lateral wall. The tunnel had opened by 10 DAB in the middle turn. The IP and OPs increased in length and changed orientation from 6 DAB onward. The angle formed by the pillar cells and the basilar membrane decreased, the OP angle decreasing substantially more than the IP angle, the angles reaching mature values by 12 DAB. The separation between the IP and OP bases, and the width of the IP heads did not reach adult-like values until 20 DAB.

**F. Basilar membrane.**

Quantitative data describing the maturation of the basilar membrane is given in Table 3.2. The division of the basilar membrane into the arcuate (inner) and pectinate (outer) zones was apparent at 2 DAB (see Figure 3.8 A). During maturation, the arcuate zone decreased in thickness from 50 \( \mu m \) at 2 DAB to 19 \( \mu m \) at 10 DAB and 9 \( \mu m \) at 20 DAB (Table 3.2). This decrease was in part due to constriction of the spiral vessel (which lay under the pillar cells) from, in the middle turn, 24 \( \mu m \) at 2 DAB to 9 \( \mu m \) at 10 DAB and about 3 \( \mu m \) at 20 DAB. Epithelial cells which form the tympanic cover layer, and are continuous with those below the zona pectinata at 2 DAB, also progressively decreased in thickness, thinning to a single layer by 20 DAB. The broader pectinate zone was
composed at 2 DAB of a thick tympanic layer, a single layer of epithelial cells of the organ of Corti and a thin hyaline layer sandwiched between the two (see Figures 3.8, 3.9 and 3.10).

Measurements of the total basilar membrane thickness in the zona pectinata, thickness of the hyaline mass and the thickness of the tympanic cover layer, were made and are given in Table 3.2. At 2 DAB the basilar membrane had a total thickness of about 73 μm in the middle turn, of which the hyaline mass accounted for 4.6 μm and the tympanic layer almost 42 μm. The thicknesses for the apical and basal turn were found to be similar. Between 2 and 6 DAB the total thickness increased to 85 μm in the middle turn, 81 μm in the apical turn and 77.8 μm in the basal turn. In all turns the hyaline mass accounted for around 16 μm of these values, it being slightly larger in the apical turn, and with slight variations in the thickness of the tympanic layer. Between 6 and 8 DAB little change occurred. From 8 DAB onward the total thickness of the basilar membrane began to decrease in the middle and apical turns. The thickness of the basal turn basilar membrane did not begin to decrease until 10 DAB. The decrease in total thickness was more marked in the apical turn, which became the thinnest of the three turns, whilst the basal turn basilar membrane decreased in thickness at a slower rate. Between 10 and 20 DAB the total thickness decreased from 68 μm to 45 μm in the apical turn, 73 μm to 56 μm in the middle turn and 81 μm to 56 μm in the basal turn. The decrease in thickness was attained through the loss of cells forming the tympanic cover layer, which more than compensated for the increase in the thickness of the hyaline mass. The hyaline mass increased in thickness progressively from 2 DAB onward and by 10 DAB had reached over 85% of its adult dimensions, which was not attained until 20 DAB. The thinning of
the lower tympanic layer did not commence until between 6 and 8 DAB, losing two thirds of its thickness by 12 DAB. The mature single cell thickness was not attained until 20 DAB.

Summary.

Both the inner and outer zones of the immature basilar membrane were characterised by the presence of a thick layer of epithelial cells on the tympanic side. The outer (pectinate) zone consisted of a single layer of organ of Corti cells, a thin layer of hyaline material and a thick epithelial layer. Maturation of this region occurred from 2-20 DAB and proceeded by a thickening of the hyaline layer accompanied by a thinning of the tympanic layer to ultimately a single cell thickness. The basilar membrane did not attain its mature appearance until 20 DAB.

3.2.2. The outer hair cell.

The OHC membrane may be divided into four regions, the apical surface, the apical part of the lateral membrane encompassing the junctional area, the lateral membrane (LM) and the basal membrane, including the nerve terminals as shown in Figure 3.20 A. Data was collected from OHCs from the apical, middle and basal turn regions of the cochlea. The membrane characteristics of the mature OHC are described initially for comparative purposes.

A. The mature OHC.

Figure 3.20 A shows a mature gerbil OHC from the middle turn and the membrane regions to be described in the study are indicated. The apical surface of the mature OHC is free from microvilli. The junctional complex around the neck of the cell may be divided into two regions. The apicalmost part comprises of up to 6 junctional strands
running parallel to the cell apex (white arrow in Figure 3.20 B); below this is a region comprising numerous strands which run perpendicular and at angles to the cell apex, extending basalward to form irregular polygonal arrays (black arrow in Figure 3.20 B). In the mature OHC, the total junctional area extends about 1.8 μm basal from the apical edge of the cell, with the parallel rows accounting for around 0.3 μm of this distance (Table 3.3). In thin sections, densely staining material is associated with the junctional region on the cytoplasmic side (Figure 3.21), and appears more extensive around the basalmost junctional area. The cuticular plate extends no further than the basalmost extent of the junctional region, and appears to contact the dense staining junctional region, although in some samples a region containing membrane bound vesicles separates the junction and cuticular plate (Figure 3.21). The lateral cisternal network commences immediately below the junctional region. Up to six layers of cisternae are present in middle turn OHCs (Figure 3.22 A) with fewer layers present in basal turn OHCs. In freeze fracture replicas of mature OHCs, the fracture plane occurs predominantly through the cisternal network, exposing only small areas of plasma membrane (Figure 3.22 B). Those areas of plasma membrane which are visible are densely packed with IMPs (Figure 3.22 B), with a mean of density of 5577 particles per μm² (Table 3.4). Regularly arranged pillar structures contact the plasma membrane and are thought to be associated with the cytoskeletal lattice. These are seen as rows of spots in tangential sections of the lateral membrane (Figure 3.22 C). The base of the mature OHC is contacted by large efferent synapses. Freeze fracture replicas reveal concentrations of particles on the OHC membrane face, the presynaptic membrane of the OHC, and indentations which may be indicative of afferent nerve terminals are also present along with the larger efferent terminal (Figure 3.23).
B. The maturation of the OHC: The apical surface.

At 2 DAB, the apical surfaces of the OHCs has a kinocilium, defined rows of stereocilia and large numbers of supernumary microvilli on the inner aspect. Each stereocilium is connected to its neighbours by 5-6 crosslinks and tip-links between stereocilia are also in evidence (Figure 3.24 A). The cuticular plate has not fully formed and microtubules running along the base of the cuticular plate region parallel to the apical surface were evident (Figure 3.24 B). Freeze fracture replicas revealed the membrane surface to be covered with many pits, possibly the openings of endocytotic vesicles (Figure 3.25 A), since thin sections of the apical region of the cell showed numbers of coated vesicles to be present in the region below the apical membrane (Figure 3.25 B).

At 8 DAB, the cuticular plate did not extend fully to the lateral membrane in all cochlear turns. In this region between the junction and the edge of the cuticular plate were accumulations of vesicles, some of which presumably correlate with the pits seen in freeze fracture replicas. Many of these vesicles formed in tracts closely associated with microtubules arising from the basal body of the kinocilium (Figure 3.26 A). The number of microvilli on the apical surface decreased between 2 and 6 DAB, by 8 DAB basal turn OHCs had lost the majority of their microvilli (Figure 3.26 B). However, microvilli remained on apical turn OHCs, consistent with a base to apex maturation of OHCs. At 12 DAB, the microvilli had regressed (Figure 3.26 C) and the cuticular plate appeared to have attained a mature configuration. By 16 DAB the apical surface resembled the adult form, with no microvilli and a greatly reduced number of vesicle openings, which were restricted to the extreme edges of the apical surface (Figure 3.26 D). Some OHCs from the apical turn showed evidence of the reabsorbed kinocilium in freeze fracture
replicas.

Summary.
Maturation of the OHC proceeded from basal turn to apical turn. The kinocilium had regressed by 16 DAB, the microvilli having been reabsorbed by 12 DAB. Pits seen in freeze fracture replicas were found in large numbers in immature OHCs, their number decreasing during maturation.

C. Junction formation.
In thin sections, the junctional region at 2 DAB appeared as a restricted, densely staining area between the OHC and supporting cell apices (Figure 3.27 A). This had increased in size by 6 DAB, with evidence of supporting cell microtubules being associated with the basal region of the junction (Figure 3.27 B). Microtubules accompanied by vesicles were evident in the basal region of the junction at 8 DAB, with the apical region of the junction having fewer associated organelles (Figure 3.27 C). The fluid spaces around the OHCs began to open around 10 DAB (see Figure 3.9), by which time the junctional region extended to the base of the head of the supporting cells (Figure 3.27 D). Supporting cell microtubules were clearly associated with the basal region of the junction. By 16 DAB, the junction appeared to have attained a mature configuration (Figure 3.27 E).

Immature junctions forming the boundaries between OHCs and supporting cells were found in freeze fracture replicas at 2 DAB (Figure 3.28 A). These junctions were much less extensive and lacked the complexity seen in the adult. However, the two distinct patterns of tight junctional strands seen in mature OHCs were identifiable at 2 DAB. In the apicalmost junctional region, one, occasionally two, junctional strands were present
running parallel to the apical surface of the cell. The basal junctional region was particularly undeveloped at 2 DAB, being less than 50% of the adult junctional depth (Table 3.3). The strands of the basal region were discontinuous and did not show the extensive polygonal formation seen in the mature OHC. The junctional strands were characterised by having large, more widely spaced particles than seen in the adult.

The rate of development of the junctional region after 2 DAB was rapid. By 4 DAB the complexity had increased, with 3-4 parallel elements present apically and the perpendicular arrays of the basal junctional region extending almost to their mature depth. The number of strands was, however, much less than in the adult (Figure 3.28 B). By 8 DAB the apical junctional region of OHCs from the middle turn had reached its mature complement of 5-6 rows of junctional strands (Figure 3.29 A). The basal region of the junction continued to increase in complexity beyond 8 DAB, and did not attain its mature configuration until 16 DAB (Figure 3.29 B, compare with Figure 3.20 B).

The complexity of the junctional region was assessed in two ways. The density of strands per μm and the number of branching points per unit area of junction were measured to give a complexity index (Table 3.3). The depth of the junction was also measured. At 2 DAB the total junctional depth was 0.72 μm, by 8 DAB the depth was 1.04 μm and had reached 1.4 μm by 10 DAB. The junctional depth was almost 1.7 μm at 16 DAB, which represented 95% of the mature junctional depth. In the basal region of the junction, the number of branching points per square μm at 2 DAB was 27, this increased to 48 by 6 DAB and to 97 by 16 DAB. The number of tight junction strands was measured and from an average of 4.8 strands at 2 DAB, this increased to 12.0 by 8 DAB and had increased
further to 14.5 by 16 DAB.

Summary.

Immature junctions were found around the neck of OHCs at 2 DAB. They consisted of a few junctional strands running parallel to the apical surface and incomplete strands running perpendicular and at angles to the apical surface down the lateral membrane. The apicalmost junction matured rapidly, with increasing numbers of parallel-oriented stands, and had an adult appearance by 8 DAB. The basalmost region continued to increase in complexity and did not appear mature until 16 DAB.

D. The Lateral Wall: Sub surface cisternal network.

The structure of the lateral membrane of adult gerbils is shown in Figure 3.22. Since OHCs from middle turns of the mature cochlea have the greatest number of cisternal layers (Forge 1991), OHCs from this turn have been chosen to illustrate the maturational sequence of the lateral cisternae.

At 2 DAB there were substantial differences in the membrane structure to that seen in the mature OHC. There was no evidence of a cisternal network, and in freeze fracture replicas, the fracture plane occurred through the plasma membrane (Figure 3.30 A). The freeze fracture replicas of the lateral membranes at 6 DAB failed to produce evidence of cisternae in OHC from any turn (Figure 3.30 B) but by 8 DAB, small isolated regions of cisternae began to be exposed in the replicas (Figure 3.30 C). At 10 DAB increasing areas of cisternae were exposed (Figure 3.31 A), the cisternal layers being clearly visible in freeze fracture replicas across the OHC membrane, together with the intercellular spaces which appeared at this time (Figure 3.31 B). By 12 DAB, the freeze fracture plane increasingly occurred through areas of cisternae, until at 16 DAB freeze fracture replicas
exposed large areas of cisternae with a corresponding decrease in regions of plasma membrane being exposed (Figure 3.31 C).

The development of the cisternal layers was followed in thin sections of OHCs. At 6 DAB there was no evidence of cisternal structures (Figure 3.32 A), and by 8 DAB thin sections showed the presence of a single cisternal layer with some regions of two layers, the innermost layer being particularly fragmentary (Figure 3.32 B). A single complete cisternal layer was present at 10 DAB (Figure 3.32 C) and by 12 DAB the two outermost cisternal layers were continuous, widely spaced pillars between the plasma membrane and the outermost layer of cisternae were evident at this stage (Figure 3.33 A). Sections from OHCs at 14 DAB showed further cisternal layers being added on the intracellular aspect (Figure 3.33 B), whilst the pillars increased in number and became more closely spaced (Figure 3.33 B). The number of pillars continued to increase through 14 DAB until 16 DAB by which time the cisternal/pillar network had taken on an adult-like configuration (Figure 3.33 A, compare with Figure 3.22 A). Higher power views of the lateral membrane show broken lengths of cistern present at 8 DAB, with no pillar structures evident (Figure 3.34 A), however the first evidence of pillars was seen at 10 DAB (Figure 3.34 B). Tangential sections of the lateral membrane showed an increase in the number and a decrease in the spacing of the pillars between 12 DAB and 16 DAB (Figure 3.34 C and D respectively). Measurements of the spacing of the pillars (Table 3.4) showed that the inter-pillar distance decreased from 53.9 ± 8.2 nm at 10 DAB, to 44.4 ± 7.01 nm at 12 DAB and to 38.6 ± 6.2 nm at 16 DAB. This compares to a mean pillar spacing of 35.4 ± 5.03 nm found in mature OHCs.
Summary.

No evidence of a sub-cisternal network was found prior to 8 DAB. The network first appeared as a single row which was not continuous until 10 DAB. Further cisternal layers were added on the cytoplasmic side of the lateral wall, the mature number being attained by 16 DAB. Pillar structures between the plasma membrane and the outermost cisternal layer did not appear until 10 DAB, which coincided with the opening of the spaces around the OHCs, increasing in number and decreasing in separation until adult-like values were reached at 16 DAB.

E. The Lateral Wall: Intra-membrane particles (IMP).

In contrast to freeze fracture replicas of the lateral membranes of mature OHCs (Figure 3.22 B), at 2 DAB, prior to the formation of the cisternal layers, the fracture plane occurred through the plasma membrane. Large areas of membrane were exposed (Figure 3.35 A), with the membrane face exhibiting both large and small particles interspaced with regions of bare membrane. Counts of the density of IMPs from middle turn OHCs were made (Table 3.4), in order to be able to directly relate IMP density changes with sub-surface cisternal layer increases. The total number of IMPs at 2 DAB was found to be 2200 per $\mu m^2$ (Table 3.4). Between 2 and 8 DAB, the fracture predominantly occurred within the plasma membrane. Over this time period, increasing numbers of IMPs and a corresponding decrease in the areas of bare membrane were seen as development progressed. Although there did not appear to be a systematic organisation to the position of these particles, concentrations of large particles were found in the region of pits which were present in the membrane fracture face (Figure 3.34 B). By 6 DAB (Figure 3.35 B), the mean IMP density had increased to 3362 per $\mu m^2$, by 8 DAB (Figure 3.35 C) this had increased to 4131 per $\mu m^2$ and by 10 DAB (Figure 3.36 A) the IMP count had
reached 4275 per μm². Throughout the period 10-16 DAB, the density of the IMP continued to increase until by 16 DAB (Figure 3.36 B) an almost mature density of 5514 per μm² had been attained (Table 3.4). Figure 3.37 shows the increasing number of IMPs plotted against the post natal age. The mean rate of increase in IMPs per square μm between 2 and 8 DAB is about 320 IMP per 24 hours and between 10 and 16 DAB is 206 IMP per 24 hours. There appears to a period between 8 and 10 DAB in which the rate of IMP addition falls to around 70 IMP per 24 hours.

Summary.

The lack of a sub-surface cisternal network prior to 8 DAB resulted in large areas of plasma membrane being exposed in freeze fracture replicas before this stage. At 2 DAB the membrane was covered in large numbers of IMPs distributed homogeneously. The numbers of IMP increased throughout the period 2-16 DAB when an adult-like IMP density was found. The rate of IMP inclusion was at its greatest between 2 and 8 DAB. The increase in numbers of IMP occurred before the appearance of the cisternal network, and before the opening of the intra organ of Corti spaces. The rate of IMP inclusion fell as the spaces appeared, about 10 DAB, but increased again between 12 and 16 DAB.

F. Basal Membrane

Whilst the mature OHC basal membrane is contacted by large efferent synapses (Figure 3.23), at 2 DAB the basal region of the OHC is only contacted by afferent fibres (Figure 3.38 A and C). The afferent synapses are recognisable in freeze fracture replicas by the presence of characteristic pits in the OHC membrane, sites of vesicle opening, presumably releasing neurotransmitter. Where the fracture face presented the neural membrane, discrete concentrations of large particles were visible (Figure 3.38 B). By 6 DAB little change had occurred with up to six afferent fibres coursing along the OHC
rows. There was little evidence of fibre tracts crossing hair cell rows at this stage (Figure 3.39 A). Figure 3.39 B shows a freeze fracture replica of the base of a 6 DAB OHC, with the afferent fibre tract completely covering the base of the cell. The concentrations of particles characteristic of afferent synapses are seen in Figure 3.39 C. At 8 DAB, the OHC was still contacted by large numbers of afferent fibres (Figure 3.40 A), but by 12 DAB small efferent fibres were also present (Figure 3.40 B). Afferent fibres still retained synapses with the OHC. However, these appeared to be decreased in area (Figure 3.40 B). By 16 DAB, large efferent terminals were present (Figure 3.41 A), covering large areas of the OHC base. Characteristics of efferents in freeze fracture replicas were seen, with lower concentrations of particles in the efferent membrane together with concentrations of particles on the OHC membrane face (Figure 3.41 B).

Summary.

No evidence of efferent innervation on OHCs was found at 2 DAB. Large numbers of afferent fibres contacted the OHCs between 2 and 12 DAB, by which time small efferent synapses were evident. Large efferent terminals were found on the OHC bases by 16 DAB.

3.2.3. The inner hair cell.

This section describes the maturation of the apical and lateral membrane of the IHC together with the junctional region around the neck of the cell. Section A describes the characteristics of the adult IHC in order to provide a context for the results presented in section B.

A. The mature IHC.

Figure 3.42 shows a mature IHC, with the four membrane regions shown. The apical membrane is free of microvilli and there are very few pits on the membrane surface seen
in freeze fracture replicas (Figure 3.43 A). A well defined junctional region was present around the neck of the cell, composed of up to 8 junctional strands separated by about 0.024 \( \mu \text{m} \) running parallel to the apical surface of the cell and a more basal region of strands running perpendicular and at angles to the apical surface (Figure 3.43 B). The total junctional depth was approximately 1.1 \( \mu \text{m} \), of which the apical region accounted for about 0.3 \( \mu \text{m} \) (Table 3.5). The complexity of the basalmost junctional region, the number of branching points per \( \mu \text{m}^2 \), was measured at 133 (Table 3.5). The lateral membrane was covered by large numbers of IMPs. Rows of large IMPs, with a mean diameter of approximately 10 nm and mean separation of 24 nm, were present against a background of more homogeneous smaller IMPs (Figure 3.44 A). The rows were occasionally in parallel (Figure 3.44 B), with a mean spacing between rows of 40 nm. A number of square arrays of particles, or plaques, were identified on the lateral membrane. These were distributed over the surface of the lateral membrane, mainly toward the apical pole of the IHC, and did not appear associated with the particles formed into rows.

**B. The maturation of the IHC: The apical surface.**

An SEM view of the apical surface of the IHC at 2 DAB is shown in Figure 3.45 A. Freeze fracture replicas (Figure 3.45 B) of the apical surface at 2 DAB show, in addition to the kinocilium, stereocilia and microvilli which appear larger and more mature than those seen on OHC at the same stage, a number of pits in the membrane. These were restricted to the strial side of the cell and were fewer in number than those seen on OHCs. These pits decreased in number and were absent by 16 DAB. In thin sections, vesicles appeared to be associated with the region of the apical membrane where the pits were seen (Figure 3.45 C), suggesting they may be the sites of vesicle opening.
C. Junction formation.

In freeze fracture replicas, immature junctions around the neck of the IHC were present at 2 DAB. These comprised an apicalmost tight junction region of 2-3 junctional strands parallel to the apical surface of the cell. Below this, strands form polygonal arrays with some strands ran parallel before turning basalward to become almost perpendicular (Figure 3.46 A). The total junctional area occupied approximately 47% of the adult junctional depth, the parallel strands extending approximately 0.05 μm of the total junctional depth of 0.5 μm (Table 3.5). By 6 DAB the parallel strands had increased to 4-5. The separation of the parallel strands had a mean of 24.5 ± 0.6 μm, the apicalmost junctional region occupying about 0.08 μm of the total mean junctional depth of 0.77 μm (Table 3.5). The basalmost region had increased in complexity and depth by this stage. The areas of membrane between the strands forming the polygonal arrays were generally free from particles (Figure 3.46 B). By 8 DAB, the apicalmost region comprised up to six strands running parallel to the surface (Figure 3.47 A). For short lengths, the number of strands running parallel increased to up to 10; however after coursing parallel to the apical surface for a short distance these strands turned basalward to course almost perpendicular to the apical surface and formed part of the polygonal array of the basal junctional region (Figure 3.47 A). By 10 DAB the complement of apicalmost strands had reached adult-like values of 6-8 strands. These were complemented by additional strands which coursed in a more complex pattern just above the basalmost junctional region, making a precise measurement of the relative depths of the apical and basalmost junctions difficult (Table 3.5). The mature junctional form was seen by 16 DAB, the total junctional depth being about 1.5 μm, with the basalmost region having resolved into a discrete region bounded apically by the parallel strands and
basally by junctional strands which oriented themselves also parallel to the apical surface, leaving no "loose ends" of junctional strands extending below this feature (Figure 3.47 B).

Measurements of the complexity of the junctional region were made. The density of junctional strands per $\mu m^2$ and the number of branching points per unit area were determined (Table 3.5). At 2 DAB the number of branching points per $\mu m^2$ in the basalmost junctional region was approximately 36, rising to 70 by 6 DAB, 109 by 10 DAB and attained an adult-like value of 129 by 16 DAB (Table 3.5). The number of junctional strands crossing the randomly drawn lines were found to increase from a mean of 5.17 at 2 DAB, to 15.3 at 10 DAB, which represented 88% of the adult-like found at 16 DAB of 17.3.

Summary.

Immature junctions consisting of a few rows of junctional strands running parallel to the apical surface and strands running basalward down the lateral membrane were present at 2 DAB. By 10 DAB the apicalmost junctional region had increased in complexity, with some parallel strands running continuously around the neck of the cell, some of which descended into the basalmost junctional region. These strands remained parallel to each other and did not appear to connect with the junctional strands which comprised the basalmost junction. This pattern of junctional arrangement differed from that seen in the OHCs. The basalmost region did not attain a mature configuration until 16 DAB whilst the number of junctional strands forming the apicalmost region did not increase after 10 DAB.
D. The lateral membrane.

Freeze fracture replicas of immature IHCs exposed large areas of the plasma membrane which enabled counts to be made of the density of IMPs on the IHC lateral wall (Table 3.6). The postnatal increase in the IMP density is shown graphically in Figure 3.48.

At 2 DAB the membrane was covered with a large number of particles, these IMPs numbering about 973 per $\mu$m^2 (Table 3.6). The total population was composed of large and small particles distributed homogeneously over the membrane, the smaller particles appearing to outnumber those of larger diameter (Figure 3.49 A). By 4 DAB (Figure 3.49 B) the IMP density had increased to a mean value of 1140 per $\mu$m^2. The larger particles had begun to organise themselves into discrete rows, although the majority of IMPs continued to appear randomly arranged (Figure 3.49 C). The distance between those larger particles comprising recognisable rows had a mean of 21.5 nm, the particles themselves having a diameter of approximately 11 nm. These values were found to be the same as for the IMP rows found in the mature IHC. By 6 DAB (Figure 3.49 D) the total number of IMPs had increased to a mean of 1342 per $\mu$m^2, the number of particles arranged in rows having also increased by this stage (Figure 3.50 A). The total IMP count increased to 1804 per $\mu$m^2 by 8 DAB (Table 3.6) and by 10 DAB the number of IMPs had reached 2020 per $\mu$m^2 (Figure 3.50 B). The lateral membrane was by this stage very densely covered with IMPs, with rows of large particles against a background of numerous smaller IMPs. From 10 DAB onward however, a substantial reorganisation of the membrane occurred. By 14 DAB the total number of IMPs had fallen to 1687 per $\mu$m^2 and by 16 DAB had fallen further to a mean of 1255 per $\mu$m^2. This decrease was due to a decline in the number of the smaller diameter IMPs, since the larger IMPs had
increased in number and in doing so creating more IMP rows. The membrane therefore appeared as a large number of large particles organised mainly in rows against a background of smaller particles (Figure 3.50 C). In regions where the particle rows ran almost parallel, the inter-row spacing had a mean of $41 \pm 2.2$ nm.

Evidence of immature plaque structures was first seen at 8 DAB (Figure 3.51 A). Although isolated examples of small plaques of IMPs were occasionally seen between 8 and 14 DAB (Figure 3.51 B), there was no evidence of a gradual increase in size or number of the plaques throughout the period. Some 16 DAB specimens had several plaques (Figure 3.51 C), however they remained substantially less prevalent than in the adult.

Summary.

IMPs were distributed homogeneously over the IHC lateral membrane at 2 DAB. The population consisted of large numbers of small particles and a smaller number of large particles. Between 2 and 10 DAB the total number of particles increased, by 10 DAB the membrane was covered with large numbers of small particles together with larger particles, some of which were organised in rows. These rows had first appeared at 6 DAB. By 14 DAB the total number of particles had decreased, in particular the number of small particles had declined. By 16 DAB an adult-like appearance had been attained, with large numbers of large particles arranged in rows and smaller particles distributed about the membrane. No evidence of plaque structures was seen prior to 10 DAB. Isolated plaques were seen at 10 and 14 DAB, and at 16 DAB they were still less in number than seen in the adult.
3.3 LABELLING OF THE TIGHT JUNCTIONAL PROTEIN ZO-1 IN THE RETICULAR LAMINA.

3.3.1 Introduction.
Whole mounts of organ of Corti tissue were prepared for fluorescent labelling of antibodies to the tight junctional protein ZO-1. The purpose of this study was to confirm the presence of this junctional component in the gerbil organ of Corti and to determine whether the antibody labelling of the tight junctions of the reticular lamina accorded with the presence of tight junctional strands described in the preceding section.

3.3.2 Methods.
Young gerbils 0, 2, 8 and 10 DAB (at least 2 in each age group) and 6 adult animals were killed with an anaesthetic overdose or in a CO₂ chamber. The bullae were quickly extracted, the cochleae dissected out and holes made in the apex and lateral wall of the cochleae. Fixative was gently perfused through the round window. The fixative was 4% paraformaldehyde in 0.1 M Phosphate buffered saline (PBS). The cochleae were then immersed in fixative under gentle rotation at room temperature for 1 hour. The organ of Corti was dissected out under 0.1 M PBS and the tissue placed in 0.3% Triton X-100 in 0.1 M PBS for 30 minutes. The segments of organ of Corti were then placed in a block solution for 1 hour at room temperature. The block solution consisted of 5% horse serum, 100 mM L-Lysine and 0.3% Triton in 0.1 M PBS. The samples were incubated in the primary antibody (1:200 of anti-ZO-1 rabbit polyclonal in 0.1 M PBS, Zymed Laboratories) overnight at 4° C. Control samples were placed in 1:200 normal rabbit serum in 0.1 M PBS overnight at 4° C. After 10 washes in 0.1 M PBS the samples were incubated at room temperature for 1 hour in the dark with the secondary antibody (1:20
TRITC swine anti-rabbit in 0.1 M PBS, DAKO). After 5 washes in 0.1 M PBS, the segments of organ of Corti were mounted on glass slides with a drop of SlowFade (Molecular Probes Inc.), coverslipped and sealed. The samples were examined under a Nikon Optiphot-2 microscope equipped for fluorescent illumination.

3.3.3 Results.

Positive immunolabelling for the ZO-1 junctional protein was found in 2 DAB gerbil organ of Corti wholemount preparations. In Figure 3.52, all cell-cell junctions of the organ of Corti and other regions forming the reticular lamina showed labelling at the apical pole of the cells. The fluorescent band was only in focus for a very restricted depth, suggesting the labelling was restricted to a narrow band around the apical end of the cell. The boundaries of the cells were well defined, with three OHC rows, a single IHC row, inner pillar cell heads and Dieters'cells being clearly identifiable. The highly regular and ordered appearance of the apical surface of the immature organ of Corti, with sensory and supporting cells in close contact was evident (Figure 3.52). A similar intensity of fluorescence was seen in apical, middle and basal turn preparations (Figure 3.52 A-C), and there appeared to be a more intense staining of the IHC junctions on the limbal side of the cell. By 8 DAB, the expansion of the supporting cells seen in SEM views of the organ of Corti was evident (Figure 3.53 A), the labelling again emphasising the high degree of regularity of the hair cell rows. In the adult, ZO-1 continued to be localised at the apical pole of the cells of the reticular lamina (Figure 3.53 B). No quantitative measures of the intensity of fluorescence was carried out, although in adult subjects the focal depth of the fluorescent band did not appear substantially greater than in 2 DAB preparations.
Summary.

The tight junctional protein ZO-1 was found to be localised at the junctions at the apical pole of hair cells and supporting cells of the reticular lamina from 2 DAB onward. The finding of positive labelling for ZO-1 at 2 DAB accords with the morphological results suggesting that junctional arrangements characteristic of tight junctions are present at 2 DAB.

3.4 THE POSTNATAL MATURATION OF THE STRIA VASCULARIS.

3.4.1. Introduction.

This section comprises a study of the onset and maturation of the endocochlear potential recorded from the scala media, an immunohistological study of Na^+-K^+ ATPase reactivity in the stria during maturation and a morphological study of the maturation of junctional complexes of marginal and basal cells of the stria vascularis.

3.4.2. Methods.

A. Morphological studies.

The methods employed for the fixation, dissection and preparation of stria vascularis for thin sections, freeze fracture and subsequent light and TE microscopy were as described in the Methods chapter. The additional methods used in the study of the maturation of the stria and measurement of the endocochlear potential are described in this section.

Measurements of intercellular junctions of the maturing stria vascularis were made from calibrated TEM prints of freeze fracture replicas using the VIDS V image analysis system. A schematic representation of the stria is shown in Figure 3.54, with the area
from which measurements were taken indicated. Measurements of the apical junctional region of marginal cells were made from prints of calibrated magnification 40k, the total junctional depth and complexity being determined as described above for hair cell apical junctions. The density of IMPs on the lateral walls of marginal cells was also calculated as described above for hair cells. Measurements of the area of gap junctions on basal cells of the stria were made from calibrated TEM prints of freeze fracture replicas. The density of the gap junctions as a percentage of the cell membrane was also calculated using a standard area of basal cell membrane over all samples. For tight junctions of the basal cells, the complexity of the junction was determined by the number of junctional strands per $\mu m^2$ from TEM prints. Standard errors for all measurements were calculated.

B. $Na^+-K^+$ ATPase reactivity.

The method of Kobayashi et al (1987) was used for the localisation of $Na^+-K^+$ ATPase reactivity. This method detects the potassium dependent p-nitrophenyl phosphatase (K-NPPase) activity of the $Na^+-K^+$ ATPase complex and not merely the presence of the enzyme. In addition, the reaction is inhibited by ouabain and is particularly useful since it occurs at physiological pH levels. In this method, the accumulation of K-NPPase in the reaction precipitate is proportionally related to the enzyme concentration, these reaction products being visualised under TEM as electron dense deposits.

Cochleae from which the stria vascularis were to be studied for localisation of $Na^+-K^+$ ATPase were dissected free of the bulla. Holes were made in the apex and lateral wall of the cochlea and the round window punctured. Fixative (2% paraformaldehyde/ 0.05% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) was perfused in through the round window, the whole cochlea then immersed in fixative under gentle rotation for 1 hour at
room temperature. Following washing with 0.1 M cacodylate buffer, the stria vascularis was dissected out under buffer. The strial tissue was divided into short lengths and washed in 50 mM Tricine, pH 7.5, for 15 minutes. Tricine buffer was used since it protected against precipitation due to non-enzymatic reactions. Samples were then placed in the incubation medium (solution A). The incubation medium contained 50 mM Tricine buffer (pH 7.5), 2 mM cerium chloride (acting as a capture agent) 10 mM magnesium chloride, 50 mM potassium chloride, 2 mM p-NPP (substrate), 5% sucrose, 2.5 mM levamisole (an inhibitor of alkaline phosphatase which can hydrolyse p-NPP) and 0.00015% Triton X-100 (to facilitate entry of the incubation medium into the tissue). The samples were incubated at 37° C. for 1 hour, then washed with 0.1 M Tris-maleate (pH 6.0, 138 mOsM) twice for 10 minutes each to remove non-specific reaction precipitates. The tissue was then washed in 0.1 M cacodylate buffer, post fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1.5 hours and then processed for TEM as described previously.

Segments of stria vascularis were also incubated in control solutions. These comprised a substrate free medium (solution B), a medium containing 10 mM ouabain (solution C) and a solution containing 50 mM choline chloride in place of KCl (solution D). The results of a control experiment from an adult gerbil are shown in Figure 3.55, with densely staining enzymatic reactivity only present in the sample containing the complete medium (Figure 3.55 A).

C. Recording of Endocochlear potential.

i. Anaesthesia and surgical procedures.

A total of 29 gerbils between 8 and 20 DAB, a minimum of 4 animals per age group and
10 adult gerbils were used to determine the onset and maturation of the endocochlear potential recorded from the scala media. Animals were anaesthetised and monitored as described in the Methods chapter above. Access to the cochlea was gained as described previously.

ii. Recording of EP.

Microelectrodes were pulled from glass micropipettes (Clark Electromedical) using a Sutter model P30 electrode puller. The tip diameters were broken back to be in the range 2-5 μm. The microelectrodes were filled with filtered 3 M KCl and inserted into a microelectrode holder (World Precision Instruments), which was also filled with 3 M KCl. The microelectrode holder was then placed in the headstage of a Neurolog NL102 D.C. preamplifier which in turn was mounted on a manually operated micromanipulator. The recorded signals were amplified by a Neurolog NL102 D.C. preamplifier and the signal displayed on a dual channel storage oscilloscope (Gould 1425). The electrode was advanced through the round window under visual control and then advanced "blind" toward the organ of Corti. The voltage changes during the penetration were recorded on the storage oscilloscope and printed out on a Gould 6120 plotter. Immediately following penetration the system was zeroed in the perilymph and the microelectrode was then advanced through the region of the organ Corti and into the scala media. The microelectrode was held in position for several minutes in order to record a stable EP value. Once in situ, the resistance of the microelectrode was measured, resistances in the range 50-90 MΩ being acceptable. Changes in the microelectrode resistance during the course of the experiment were taken as indicators of broken or blocked electrode tips, in which case the microelectrode was replaced.
3.4.3. Results.

A. The mature stria vascularis.

Figure 3.55 A-D shows thin sections through the mature stria vascularis of the gerbil. The marginal cells face the endolymphatic space, the convoluted lateral wall being created by infoldings of the lateral membrane. Processes extend deep into the body of the stria, and intense staining for Na\(^+\)-K\(^+\) ATPase reactivity was seen on the basolateral membrane (see Figure 3.55 A). In freeze fracture replicas, the infoldings of the lateral membrane were covered by large numbers of intramembrane protein particles (see Figure 3.56 B), which may be related to the presence of Na\(^+\)-K\(^+\) ATPase. Elongated basal cells lie below the marginal and intermediate cells.

Figure 3.56 A shows a freeze fracture replica of the marginal cell tight junctional region from an adult. The mean total junctional depth was 0.98 \(\mu\)m, the parallel arranged tight junctional strands numbered between 6 and 8, with a complex region of strands extending below this. The number of branching points per \(\mu\)m\(^2\), a measure of the complexity of the junction, was approximately 100 (Table 3.7). The lateral membrane of the marginal cell is covered with large numbers of IMP (Figure 3.56 B), possibly related to sites of the Na\(^+\)-K\(^+\) ATPase enzyme. The adult basal cell tight junctional conformation is shown in Figure 3.57 A. Extensive tight junctions composed of long strands of closely spaced particles cover almost the whole of the membrane. A mean of 167 strands per \(\mu\)m\(^2\) was found and up to 8 strands were oriented parallel to each other in some regions (Table 3.9). Between these junctional strands were found gap junctions of varying area and which were located between the tight junctional strands in no apparent pattern (Figure 3.57 B). Large numbers of gap junctions were also present on the apical membrane of
basal cells (Figure 3.57 C). These gap junctions were within well defined boundaries and occupied a mean of 16.9\% of the total cell membrane (Table 3.9). The individual gap junctions had a mean area of 0.32 \mum^2.

**B. Gross morphological maturation of the stria.**

Thin sections through the stria vascularis from 2-20 DAB are shown in Figure 3.58 and 3.59. At 2 DAB the marginal cells were of a simple cuboidal shape, with some lipid bodies present (Figure 3.58 A). The apical surface facing the lumen of the scala media was covered in microvilli, and there was little evidence of infoldings of the lateral membrane. Junctions between marginal cells were evident at the apical pole of the cells (Figure 3.58 A arrow). Pigment containing cells, derived from neural crest cells, lay below the marginal cell layer. These will ultimately form the intermediate cells. There was no evidence of basal cells, the strial tissue lying on a basal lamina. By 6 DAB, the infolding of the lateral membrane of the marginal cells had greatly increased. The intermediate cell layer began to be invaded by extensions of the marginal cells (Figure 3.58 B) and elongated basal cells were present below the intermediate cell layer. By 12 DAB the capillaries had migrated into the body of the stria, and the intermediate cells were engulfed by the extensive processes from the marginal cells (Figure 3.59 A). Basal cell processes which entered the intermediate cell layer from below were also present. By 20 DAB the marginal cell processes extended deep into the stria, surrounding the capillaries and intermediate cells (Figure 3.59 B). The basal cells were elongated and demarcated the stria from the spiral ligament (Figure 3.59 B).

**Summary.**

Darkly staining marginal cells and the precursors of intermediate cells were present in the stria at 2 DAB. By 6 DAB the basal cells had appeared and the process of infolding
of the lateral membrane of marginal cells had commenced. By 12 DAB extensive interdigitation of the marginal cell membranes was apparent and the elongated basal cells continued to flatten. At 20 DAB the stria was adult-like in appearance, the marginal cell processes having engulfed both intermediate cells and capillaries. The basal cells were the last cell type to appear.

C. *Endocochlear potential onset and maturation.*

Figure 3.60 shows the EP trace obtained from a 20 DAB animal, showing a steady EP value of approximately 85 mv. Figure 3.61 shows the onset and rise of the EP measured from gerbils between 8-20 DAB. Between 8-10 DAB, EP was just detectable at a few millivolts (mv). The EP rose from 8 mv at 10 to 15 mv at 12 DAB, after which the rate of increase of EP accelerated. From a mean of 15 mv at 12 DAB the EP attained 48 mv by 16 DAB and a mean value of 82 mv by 20 DAB which represented 92% of the EP measured in adult animals. Between 12 and 20 DAB the EP increased at a mean rate of approximately 8.4 mv per 24 hours.

**Summary.**

The EP was first recordable at 10 DAB. Between 12 and 16 DAB the EP rose rapidly, rising less rapidly between 16 and 20 DAB. The time period 12-16 DAB coincides with the onset and rapid rise of CM, CAP and SFOAEs described earlier. Further increases in the EP level occurred from 20 DAB.

D. *Na⁺-K⁺ ATPase Reactivity.*

At 2 DAB there was evidence of low levels of Na⁺-K⁺ ATPase reactivity (Figure 3.62 A). By 6 DAB the marginal cells had begun to extend processes into the intermediate/basal cell layers and infolding of the marginal cell lateral membrane had increased. This was accompanied by an increase in the intensity of staining for Na⁺-K⁺ ATPase reactivity
By 12 DAB Na\(^{+}\)-K\(^{+}\) ATPase reactivity had continued to increase, with dense staining evident on the marginal cell lateral wall infoldings. The intensity of staining was particularly evident on the basolateral region of the marginal cell walls, where the marginal cell processes extended toward the basal cell layer (Figure 3.63 A). The intensity of staining found at 12 and 16 DAB (Figure 3.63 B) was similar to that found in the adult. A comparison of higher power views of TEM sections and freeze fracture replicas of the infoldings on the marginal cell lateral membrane showed that increased intensity of staining for Na\(^{+}\)-K\(^{+}\) ATPase reactivity at 6, 12 and 20 DAB (Figure 3.64 A-C respectively) coincided with an increase in the density of IMPs on the lateral membrane infoldings. Figure 3.65 A-C shows freeze fracture replicas of the lateral wall infoldings, showing increasing IMP density. Counts of IMP density on the lateral membrane infoldings increased from a mean of 2501 ±91.2 per μm\(^2\) at 6 DAB to 3135 ±89.9 per μm\(^2\) at 20 DAB.

**Summary.**

Na\(^{+}\)-K\(^{+}\) ATPase reactivity was detectable at 2 DAB, localised at the marginal cell lateral membrane. The intensity of the staining increased by 6 DAB, which coincided with an increase in the surface area of the marginal cells due to the beginning of infolding of the lateral membrane. By 12 DAB the intensity of staining was comparable to that of the adult. This coincided with the onset of a measurable EP.

**E. Development of the tight junctions of marginal cells.**

At 2 DAB a well defined junctional region was present at the luminal pole of the marginal cells, composed of large, widely spaced particles. The apicalmost region of the junction consisted of up to 4 strands oriented parallel to the apical surface of the cell, with a number of more irregularly arranged and discontinuous strands below these
The junction extended about 0.194 μm basalward from the apical edge of the cell (Table 3.7). By 6 DAB, the particles comprising the apicalmost junctional strands had increased in density. Up to 6 parallel strands were present in some areas, whilst below these discontinuous strands continued to extend basalward, indicating that this region in particular was still developing (Figure 3.66 B). The total junctional depth had increased to approximately 0.304 μm by this stage (Table 3.7).

From 8 DAB onward, the strands comprising the apicalmost junctional region ceased to form a ring around the neck of the cell. In place of the earlier concentric, parallel junctional strands, the strands ran parallel to the apical surface for a short distance before turning basalward, maintaining their parallel relationship, and entering into the basalmost junctional region (Figure 3.67 A). These strands formed whorl-like patterns, and became indistinguishable from the strands which had formed the well defined basalmost junctional region previously. A consequence of this reorganisation was that measurements of the complexity of the junctional region, determined by the number of branching points per μm² of junction, indicated that the complexity of the junction fell from 265 branching points per μm² at 6 DAB to a mean of 162 at 8 DAB and to a mean value of 137 per μm² at 16 DAB (Table 3.7). This was due to the apicalmost strands entering the basal region but failing to form branches and connections with other junctional strands (Figure 3.67 B). This contrasted with the multiple branching seen in strands which had earlier occupied the basalmost junctional region. Between 8 and 12 DAB the total depth of the junction increased from 0.48 μm to 0.73 μm. The number of junctional strands running parallel to the apical surface did not increase beyond 12 DAB, however the strands continued to extend basalward along the lateral membrane. By 16 DAB the total junction
0.86 µm, representing 88% of the mature junctional depth (Table 3.7).

Summary.

The junction around the apical pole of the marginal cells was well defined at 2 DAB, with junctional strands running parallel to the apical surface in a tight junctional configuration and other strands running basalward. This pattern was similar to that seen in OHC and IHCs, however the junction appeared more developed at 2 DAB, particularly in the number of parallel oriented strands. From 6 DAB the tight junctional strands descended into the basalmost junctional region, in a similar pattern to that seen in IHCs. The parallel strands appeared adult-like by 12 DAB, however the basalmost junctional region did not attain a mature appearance until 16 DAB. The formation of the mature tight junction coincided with the onset of measurable EP.

F. Tight junctions between adjacent basal cells.

At 6 DAB, strands of developing tight junction were present, composed of a few large particles, some of which were widely spaced (Figure 3.68 A). The complexity of the tight junction was measured, and the results are given in Table 3.8. The tight junctional strands had only slightly increased in number by 8 DAB from a mean of 34.7 at 6 DAB to a mean of 35.4 junctional strands per µm^2 at 8 DAB. However, the number of IMPs composing the strands had increased (Figure 3.68 B), and small aggregations of particles had begun to form between the junctional strands (Figure 3.68 B). Between 8- 10 DAB the number of junctional strands increased to a mean of 72.6 strands per µm^2 at 10 DAB (Table 3.8). The strands covered increasing amounts of the membrane and the individual strands were in closer proximity (Figure 3.69 A). By 12 DAB the junctional strands extended over almost the whole basal cell membrane (Figure 3.69 B). Parallel rows of strands had developed by 12 DAB, composed of about 4- 6 junctional strands (Figure
Some of these junctional strands enclosed areas of densely packed particles, resembling gap junctions, creating a junctional form not seen in other regions of the organ of Corti (Figure 3.70 A). Between 12-18 DAB, the tight junctional strands oriented parallel to each other had continued to increase in number. Some of these regions contained up to 8 strands, though most comprised 3 or 4 strands. The presence of broken junctional strands indicated that maturation was still proceeding (Figure 3.70 B).

Summary.

Although broken lengths of tight junctional strand were present between 2 and 6 DAB, they had not organised into large numbers of parallel strands until 8 DAB. A rapid increase in both the number of strands and the number of strands organised in parallel rows occurred between 8 and 10 DAB. By 12 DAB, the onset of EP, these strands had attained a high degree of complexity, and continued to increase in number between 12 and 18 DAB, the period of greatest EP rise.

G. Basal cell apical membrane gap junctions.

Identifiable basal cells were not present prior to 6 DAB. On freeze fracture replicas of basal cell apical membranes at 6 DAB, patches of particles had begun to appear. Differences between the apical and basal turn were evident. Apical turn gap junctions were composed of few large particles, the aggregations of particles forming irregularly shaped patches (Figure 3.71 A) whilst a freeze fracture replica from the basal turn of the same cochlea showed that the gap junctions occupied a larger area than those of the apical turn. The junctional elements were also more densely packed (Figure 3.71 B). Measurements of the area occupied by gap junctions were made compared to a defined basal cell membrane area and are given in Table 3.9. At 6 DAB the gap junctions had
a mean area of 0.01 $\mu m^2$ and occupied a mean of 6.7% of the membrane area. The junctions had increased slightly by 8 DAB to occupy about 9% of the membrane area (Table 3.9). Small, well defined gap junctions composed of tightly packed particles were present along with larger, less well defined areas of particles which were less densely packed (Figure 3.72 A). By 12 DAB the number of particles comprising the gap junction had increased dramatically, the junctional regions extending over large areas of the membrane (Figure 3.72 B). The junctions had a mean area of 0.04 $\mu m^2$ and occupied approximately 12.7% of the membrane area (Table 3.9). The junctional units continued to cover large regions of the membrane at 16 DAB, extending over approximately 26% of the membrane area, the individual junctions having a mean area of 0.25 $\mu m^2$. The "compressing" of the gap junctional units into smaller, more densely packed patches continued through 18 DAB, at which stage the gap junctions were composed of densely packed large particles, and had re-arranged into discrete patches (Figure 3.73). However, these gap junctional patches were not as regular as seen in the adult, which had well defined patches of very densely packed junctional units (see Figure 3.55 C), and had a mean area of 0.325 $\mu m^2$ occupying approximately 18.2% of the membrane (Table 3.9).

**Summary.**

Small gap junctions appeared on basal cells at 6 DAB, and occupied a small area. By 12 DAB the gap junctions had increased in area and number. The junctions extended over large areas of membrane, and continued to increase in area between 12- 16 DAB. This period marks the onset and rapid rise of EP. The gap junctions seen in the adult covered less of the membrane than at 16 DAB, but were more highly organised suggesting gap junctional maturation continued beyond 16 DAB.
FIGURE 3.1.

Maturation of stimulus evoked cochlear responses.

Cochlear microphonic potential (CM). The mean maximum CM amplitude elicited by a constant tone delivered at 75 dB SPL at the frequencies 1.5 and 4 kHz. CM was measurable at 14 DAB at both frequencies.
FIGURE 3.2.

Compound action potential (CAP) recorded at the round window to tone bursts at 1.5 kHz and 4 kHz. The mean maximum CAP amplitude in response to tone bursts at 75 dB SPL is shown and was first measurable at 14 DAB.
FIGURE 3.3.

Upper panel. The unaveraged stimulus recorded from the meatus, showing the continuous probe tone at 1.5 kHz and a suppressing tone burst at 60 and 66 dB SPL respectively.

Centre panel. The unfiltered SFOAE residual revealed after the waveform manipulations were carried out as described in the Methods.

Lower panel. Following digital filtering centred on the stimulus frequency, the residual exhibits a clear latency with respect to the tone burst in the Upper panel. This indicates an intracochlear origin for the recorded waveform.
FIGURE 3.4.

Stimulus frequency otoacoustic emissions (SFOAE). The SFOAE residual produced by a 4 kHz tone was detectable at 14 DAB, but not until 16 DAB with a 1.5 kHz stimulus tone. N = 4 in each age group for all measurements.
FIGURE 3.5.

SEM views of the apical surface of the maturing organ of Corti.

A-C) 2 DAB.

A) Apical turn. Well defined stereociliary bundles on OHCs (large arrow) and IHCs (arrowhead) are visible. Kinocilia are present on sensory cells and supporting cells (small arrows). The stereocilia of IHCs appear less well developed than those of the OHCs.

B) Middle turn. The stereociliary bundles are more developed than in the apical turn. The three rows of OHCs are equidistant, whilst IHCs form a single row.

C) Basal turn. The IHC stereocilia are well defined, appearing thicker than those of the OHCs. Kinocilia are present on the OHCs (arrow), as well as large numbers of microvilli on hair cells and supporting cells.

D-F) 6 DAB.

D) Apical turn. OHCs retain a rounded, immature appearance with kinocilia on supporting cells (arrow).

E) Middle turn. The distance between the outermost and middle rows of OHCs has begun to increase due to expansion of the supporting cells (arrow).

F) Basal turn. No expansion of supporting cells was seen.

Scale bar = 5 μm.
G-I) 8 DAB.

G) Apical turn. Microvilli and kinocilia still present on sensory cells and supporting cells.

H) Middle turn. The expansion of the supporting cells, the Deiters' cells had further increased the separation of the OHC rows, whilst kinocilia were evident on the apical surfaces of the supporting cells (arrow).

I) Basal turn. The microvilli on OHCs had begun to regress (black arrow), but were still in evidence on the IHC (white arrow).

J) 10 DAB. Immature OHCs were found in the apical turn, microvilli were still present on the apical surface inside the stereocilia.

K) 12 DAB. Immature OHCs, with the kinocilium centrally located in the stereociliary bundle, were present in the apical turn.

Scale bar G-I= 5 μm, J and K = 2 μm.
FIGURE 3.5.

L- N) 16 DAB. The reticular lamina had taken on a mature appearance. Isolated kinocilia were occasionally seen on both supporting cells (arrow) and OHCs in the apical turn (L).

Scale bar = 10 μm.
FIGURE 3.6.
Plot of the increase in the width of the organ of Corti region containing the sensory cells.
The middle turn increased in width at a greater rate than the apical or basal turns.
DAYS AFTER BIRTH — APICAL TURN

MIDDLE TURN

BASAL TURN
FIGURE 3.7.
The increase in the supporting cell head width in the middle turn. The second row of Deiters’ cells showed the greatest rate of increase in width between 6 and 8 DAB.
DAYS AFTER BIRTH

CELL HEAD WIDTH μm

- DEITERS CELL 1
- DEITERS CELL 2
- INNER PILLAR

132
FIGURE 3.8.

Light micrograph sections through the middle turn of the maturing organ of Corti.

A) 2 DAB. The hyaline layer of the basilar membrane was present as a thin layer (arrow) between the cells of the organ of Corti above and the epithelial tympanic cover layer below (t).

B) 4 DAB. The lumen of the inner spiral sulcus had begun to open (arrow) and the hyaline layer had commenced thickening (h).

C) 8 DAB. The hyaline layer had continued to increase in thickness (h) whilst the epithelial layer below the zona arcuata had begun to thin (open arrow).

Scale bar = 20 μm
FIGURE 3.9.

A-C) 10 DAB. Light micrograph sections through apical, middle and basal turns of the same cochlea.

A) Apical turn. No spaces within the body of the organ of Corti were apparent. OHCs (o) and IHC (i) are shown.

B) Middle turn. Extensive opening of the tunnel of Corti (T) had occurred and to a lesser degree spaces of Nuel were opening (N). The hyaline layer of the basilar membrane (H) and the epithelial tympanic cover layer (E) is shown.

C) Basal turn. The tunnel of Corti was not as well developed as in the middle turn, with the spaces of Nuel still to appear.

Scale bar A-C = 15μm.
FIGURE 3.10

A) 12 DAB. The epithelial layer of the zona arcuata had continued to thin (open arrow) whilst the tunnel of Corti above this had opened. The epithelial layer below the hyaline layer of the basilar membrane had also decreased in thickness, whilst the hyaline layer had increased in thickness (H).

B) 20 DAB. The epithelial tympanic layer had thinned to a single layer (arrow).

Scale bar A = 20 μm, B = 35 μm.
FIGURE 3.11.

Tectorial membrane maturation.

A) 2 DAB. The tectorial membrane was composed of loosely organised fibrils, the thicker fibrils running longitudinally (arrowhead) and radially (arrow).

B) 4 DAB. The density of fibrils had increased.

Scale bar = 5 μm
FIGURE 3.12.

A) 6 DAB. Pillar-like trabeculae contacted the OHC stereocilia (arrows).

B) 12 DAB. The tectorial membrane had taken on an adult-like appearance, the filaments composing the membrane having increased in thickness (arrows).

Scale bar = 5 μm
FIGURE 3.13.

A) 8 DAB. Microvilli (arrows) on the surface of the greater epithelial ridge contacted an amorphous layer below the fibrils composing the tectorial membrane (T).

B) 8 DAB. The tectorial membrane appears to form from material (arrows) released by the cells of the inner sulcus.

Scale bar A = 330 nm, B = 500 nm.
FIGURE 3.14.

Changes in middle turn pillar cell orientation during maturation.

A) 2 DAB. The pillar cells (P) were oriented almost vertically between the IHC (I) and OHC (O), occupying a small area of the apical surface of the organ of Corti, their apical surface covered in microvilli (arrow).

B) 8 DAB. Microtubule bundles extended further toward the base of the cell (arrow). Little enlargement of the pillar cell head had occurred.

Scale bar A = 2.5 μm, B = 1.5 μm.
FIGURE 3.15.

Changes in middle turn pillar cell orientation during maturation.

A) **10 DAB.** The inner pillar (IP) head had enlarged dramatically (arrow), extending over the top of the outer pillar (OP) cell. Microtubule bundles ran parallel to the apical surface.

B) **16 DAB.** The IP head had further enlarged, increasing the separation of the OHCs and IHC. The microtubule bundle was organised into tightly packed bundles (arrow).

Scale bar A = 3 μm; B = 2.5 μm.
FIGURE 3.16.

The opening of the spaces between OHCs and supporting cells.

A-C) 6 DAB.

A) Cross-section of the organ of Corti at 6 DAB, with the pillar cell (P) lying between the OHC (O) and IHC (I). Microvilli were present on the apical surface of the pillar cell.

B) The microtubules in the IP cell formed discrete bundles (arrows).

C) Junctional patches were present between IP and OPs (arrowheads), but were not apparent between the IHCs (I) and supporting cell (P).

Scale bar = 5 µm, B = 1.5 µm, C = 500 nm.
FIGURE 3.17.

The opening of the spaces between OHCs and supporting cells.

A-C) 8 DAB.

A) A section through the organ of Corti showed no evidence of spaces appearing.

B) At high power, a clean break (arrow) between the OHC (O) and pillar cell (P) was seen.

C) Evidence of junctions between the OP and IP (arrow) as well as the IP and OHC (O). No junctions were seen between the OP and OHC.

Scale bar = 5 μm, B and C = 200 nm.
FIGURE 3.18

The opening of the spaces between OHCs and supporting cells.

A-B) 10 DAB.

A) Extensive space opening had occurred, the space of Nuel was present (N) with the lateral walls of the IP and OPs having an irregular appearance (arrowhead). Junctional patches were present between the IP and OPs (arrow).

B) microvilli were present on the lateral walls of the IP and OPs which faced the fluid spaces (arrows).

Scale bar A = 5 µm; B = 500 nm.
FIGURE 3.19.

Increase in the length of the pillar cells in the middle turn during maturation.
DAYS AFTER BIRTH

--- INNER PILLAR

--- OUTER PILLAR
FIGURE 3.20.

Adult OHCs from the middle turn.

A) The membrane regions described in the study are indicated; AP: apical membrane, JR: junctional region, LM: lateral membrane, BM: basal membrane.

B) Freeze fracture replica of the junctional region around the neck of the OHC. The apicalmost region (white arrow) consists of up to 6 parallel strands, whilst the basal region (black arrow) consists of irregularly arranged strands with many branching points.

Scale Bar A = 3 μm; B = 200 nm
FIGURE 3.21.

Thin section of the junctional region between a supporting cell (OP) and OHC (O). The apicalmost junctional region appears tightly sealed and lacks associated material on the supporting cell side (arrow). Dense material occurs in both hair cell and supporting cell, being particularly in the basal junctional area (arrowhead). Microtubules are associated with the basalmost junctional region in the supporting cell (MT).

Scale Bar = 100 nm.
FIGURE 3.22.

A) Thin section of the lateral membrane of an OHC from the middle turn. Five cisternal layers are present (arrows) on the inside of the plasma membrane.

B) Freeze fracture replica of the lateral membrane. The fracture exposes only small areas of plasma membrane (P) densely packed with IMPs, the fracture plane occurring mainly through the cisternal layers (LC).

C) Grazing section of the lateral wall of an OHC. The pillars of the cytoskeletal lattice are seen as regularly spaced spots (arrows), arranged in rows.

Scale Bar A = 200 nm; B = 100 nm; C = 150 nm.
FIGURE 3.23.

Freeze fracture through the basal end of the OHC (O) and its associated efferent terminal (N). Large pits (arrow) in the OHC membrane replica indicate sites of neurotransmitter release at an afferent synapse. Large numbers of small particles (open arrowhead), some in densely packed plaques are also present. A large efferent terminal (N) covers the central region of the OHC base.

Scale bar = 200 nm.
FIGURE 3.24.
Apical region of 2 DAB OHCs.

A) Thin section through the apical region showing numerous microvilli (M), connected by crosslinks to their neighbours. Tip-links on stereocilia are already present (T), the associated density at the apex of some of the stereocilia (S) can also be discerned.

B) Microtubules (MT) run below the immature cuticular plate.

Scale Bar A = 300nm; B = 200 nm.
FIGURE 3.25.

A) Freeze fracture replica of the apical surface. Numbers of pits are visible (arrowheads), together with cross fractured kinocilium (K), stereocilia (S) and microvilli (M).

B) Thin section of the apical membrane. Coated vesicles (arrowhead) are apparent in the region adjoining the supporting cell junctional region.

Scale bar A = 1 μm; B = 100 nm.
FIGURE 3.26.

Apical region of the OHC 8-16 DAB.

A) 8 DAB. Thin section of the apical region. The basal body of the kinocilium (K) has microtubules (arrows) associated with it.

B) Freeze fracture replica of 8 DAB OHC apical membrane from the middle turn. The microvilli have begun to be reabsorbed. Vesicle pits are still present (arrows).

C) 12 DAB. Thin section of a middle turn OHC. The microvilli have almost totally been reabsorbed. The cuticular plate is more densely staining by this stage, extending almost to the junctional regions.

D) 16 DAB. Freeze fracture replica of an apical turn OHC. The remnant of the kinocilium is visible (arrowhead), whilst the number of vesicle pits (arrow) is much reduced compared to earlier examples.

Scale bar A = 380 nm; B- D = 1 μm.
FIGURE 3.27.

Formation of the OHC apical junctional region.

A) 2 DAB. Thin section of the junction region between an OHC (O) and pillar cell (S), seen as a densely staining area.

B) 6 DAB. The junction has increased in size. The apical most region (a) has less associated cytoplasmic elements in the supporting cell side than the basalward junctional area (b). Microtubules are associated on the supporting cell side (MT) of the basal region of the junction.

C) 8 DAB. Microtubules associated with the supporting cell form discrete tightly packed bundles (MT). Vesicles lie between the microtubules and the basal junctional area. On the OHC side, the cuticular plate (CP) extends almost to the junction. A kinocilium is present on the apical aspect of the supporting cell (K).

D) 10 DAB. The microtubule bundles (MT) of the supporting cell are associated with the dense region next to the basalmost junction region (b). The apicalmost region (a) appears more tightly sealed with less densely staining material associated on the OHC side.

E) 16 DAB. The junction appears to have a mature configuration. The basalward area (b) has densely staining material on the supporting cell side (S). The cuticular plate (CP) extends to the base of the junction area.

Scale bar A = 700 nm; B = 300 nm; C = 700 nm; D = 200 nm; E = 600 nm.
FIGURE 3.28.

Freeze fracture replicas of the developing OHC apical junction.

A) 2 DAB. The apical most region (a) is present in the form of two strands running parallel to the apical surface. The basalward area (b) consists of incomplete strands running at angles to the apical surface. The particles comprising the strands are not as tightly packed as in the mature junction.

B) 4 DAB. The apical junction region consists of up to 4 parallel strands. The basalmost area has increased in complexity, both in the number of strands and the degree of branching.

Scale Bar A = 250 nm; B = 400 nm.
FIGURE 3.29.
Freeze fracture replicas of the developing OHC apical junction.

A) 8 DAB. The apical region (a) has an almost mature form with up to 6 parallel strands. The basalmost (b) area continues to increase in size and complexity.

B) 16 DAB. Little change to the apicalmost region (a) has occurred since 8 DAB. However, the basalward region (b) has become more complex with increased numbers of strands and more complex branching patterns. The particles comprising the strands are more tightly packed than in younger examples. By 16 DAB, the junction resembles the mature form.

Scale bar A and B = 250 nm.
FIGURE 3.30.

Maturation of the lateral membrane in freeze fracture replicas.

A) 2 DAB. The fracture occurs through the plasma membrane (PM) exposing large areas of membrane, covered with scattered IMPs (arrows).

B) 6 DAB. The fracture continues to be through the plasma membrane, indicating that the cisternal network has yet to form. Depressions in the membrane are associated with groups of IMPs (arrow), possibly signifying sites of IMP incorporation.

C) 8 DAB. Large areas of the plasma membrane continue to be exposed in freeze fracture replicas, and small areas of cisternae begin to appear.

Scale Bar A and B = 350 nm; C = 150 nm.
FIGURE 3.31.

Maturation of the lateral membrane in freeze fracture replicas.

A) 10 DAB. Larger regions of cisternae (lc) are exposed, whilst areas of plasma membrane are still visible.

B) 10 DAB. A fracture across the membrane exposes the cisternal layers (lc) of two adjacent OHCs, with a narrow intercellular space (sp) intervening.

C) 16 DAB. The fracture plane occurs mainly through the cisternal layer (lc), exposing only a small area of the plasma membrane around the periphery.

Scale bar A = 150 nm; B = 350 nm; C = 200 nm.
FIGURE 3.32.

Thin sections of the developing lateral wall.

A) 6 DAB. No cisternae are present below the OHC lateral membrane.

B) 8 DAB. Broken lengths of cisternae are present (arrows) below the plasma membrane.

C) 10 DAB. A single almost continuous layer of cistern (arrow) is present in thin sections.

Scale Bar = 300 nm.
FIGURE 3.33.

A) 12 DAB. Two layers of cisternae are seen in thin sections.

B) 14 DAB. Pillar structures (arrows) are in evidence between the outer cisternal layer and the plasma membrane.

C) 16 DAB. Further cisternal layers have been added, with the pillar structures more closely spaced (arrows).

Scale bar A = 300 nm; B and C = 200 nm.
FIGURE 3.34.

A) 8 DAB. High power view of the developing first cisternal layer (arrow) below the plasma membrane. No pillar structures are visible.

B) 10 DAB. Pillar structures are visible (arrows). They are more widely spaced than in the mature OHC.

C) 12 DAB. Grazing sections of OHC membrane show the pillars in transverse section, seen as rows of spots (arrows).

D) 16 DAB. Tangential section of the OHC membrane shows a decreased spacing of pillars (arrows).

Scale bars A = 200 nm; B = 150 nm; C = 100 nm; D = 200 nm.
FIGURE 3.35.
Freeze fracture replicas of the lateral membrane: Intramembrane Particles.

A) 2 DAB. Widely spaced populations of both large and small particles are in evidence. There does not appear to be a systematic organisation to the position of these intramembrane protein particles.

B) 6 DAB. The number of IMPs has increased. Concentrations of IMPs appear around pits in the membrane (arrow).

C) 8 DAB. The IMPs continue to increase in density. Due to the increasing close packing, it was not possible to distinguish the large and small particles, however the large particles are much increased in number with respect to 2 DAB.

Scale bar = 100 nm.
FIGURE 3.36.

Freeze fracture replicas of the lateral membrane: Intramembrane Particles.

A) 10 DAB. The density of IMP packing continued to increase.

B) 16 DAB. A mature IMP density has been attained.

Scale bar = 100 nm.
FIGURE 3.37.

Plot of the increase in IMP density with postnatal age, from 2 DAB until 16 DAB, with adult (36 DAB) values shown for reference. There appears to be a period between 8 and 10 DAB when the inclusion of IMPs decreases, before increasing again to attain the mature values. Standard error bars are shown.
DAYS AFTER BIRTH

(X 1000)

OHC IMP PER Ln²

0 4 8 12 16 20 24 28 32 36
FIGURE 3.38.

Basal membrane maturation.

A) 2 DAB. Freeze fracture replica of the OHC base. Indentations on the OHC show the position of the afferent terminal, whilst pits indicate sites of vesicle release, presumably containing neurotransmitter.

B) 2 DAB. High power view of particle clusters characteristic of afferent nerve terminals.

C) 2 DAB. Thin section of the OHC (O) base, showing numerous afferent nerve terminals (a).

Scale Bar A = 700 nm; B = 150 nm; C = 300 nm.
FIGURE 3.39.

Basal membrane maturation.

A) 6 DAB. Freeze fracture replica along the bases of two rows of OHCs. Up to six afferent fibres course along the OHC bases (arrows), with no evidence of cross row fibre projections.

B) 6 DAB. Higher power view of the OHC base. Where the OHC membrane is exposed pits are evident (arrow).

C) 6 DAB. Higher power view of an afferent nerve terminal show closely packed particles.

Scale bar A = 4 μm; B = 700 nm; C = 275 nm.
FIGURE 3.40.
Basal membrane maturation.

A) 8 DAB. Thin section showing numerous afferent terminals (a) contacting the OHC. No efferent fibres are visible.

B) 12 DAB. Small efferent terminals (e) have appeared on the OHC base between afferent terminals (a) which still maintain synaptic contacts.

Scale bar A = 300 nm; B = 150 nm.
FIGURE 3.41.

Basal membrane maturation.

A) 16 DAB. Freeze fracture replica of the OHC base, showing several efferent terminals (E). These are characterised in freeze fracture by the presence of pits on the neural face.

B) 16 DAB. High power freeze fracture replica of the OHC basal membrane region showing an efferent terminal (E). Concentrations of particles on the OHC membrane face are also seen (arrows).

Scale bar A = 700 nm; B = 275 nm.
FIGURE 3.42.

Thin section of a mature IHC, showing the membrane regions described in the study; apical membrane, a; junctional region, J; and lateral membrane, LM.

Scale bar = 2 μm.
FIGURE 3.43.

Mature IHCs.

A) Freeze fracture replica of the apical surface of a mature IHC.

B) The apical junctional region of a mature IHC. The apicalmost region (a) consists of up to 6 strands running parallel to the apical surface. The basalmost junction (b) is composed of eccentrically coursing strands, the basalmost extremity of the junction is delimited by further parallel oriented strands.

Scale bar A = 1 μm; B = 200 nm.
FIGURE 3.44.

Mature IHC.

A) Freeze fracture replica of the lateral membrane of the IHC, showing rows of large particles against a background of smaller IMPs.

B) Higher power view of the lateral membrane. Rows of IMP occasionally run parallel to each other.

Scale bar A = 500 nm; B = 100nm.
FIGURE 3.45.

Apical surface of 2 DAB IHC.

A) 2 DAB. SEM view of the apical surface showing robust stereocilia (S) and large numbers of microvilli (M) which are cross-linked to their neighbours by up to 6 filaments.

B) 2 DAB. Freeze fracture replica of the apical surface of an immature IHC.

C) 2 DAB. Thin section through the apical region of an IHC. Coated vesicles appear to be associated with the apical membrane (arrow).

Scale bar A, B = 1 μm; C = 2 μm.
FIGURE 3.46.

Maturation of the IHC apical junctional region.

A) 2 DAB. Junctional strands run parallel to the apical surface, forming the apicalmost region (a), whilst broken strands form the basalmost region (b).

B) 6 DAB. The basalmost region had increased in depth, whilst the apicalmost region consisted of up to 4 parallel strands.

Scale bar = 100 nm.
FIGURE 3.47.

Maturation of the IHC apical junctional region.

A) 8 DAB. Up to 6 strands run parallel to the apical surface before coursing basally to form whorl patterns (arrows). This increases the number of strands comprising the basalmost junctional region.

B) 16 DAB. The junctional region is mature in appearance.

Scale bar = 100 nm.
FIGURE 3.48.

Plot of the changing IMP density on the IHC lateral membrane during maturation.

Standard error bars are shown.
FIGURE 3.49.

IHC lateral membrane maturation.

A) 2 DAB. Large (open arrow) and small (arrow) diameter IMPs cover the membrane with no discernable pattern.

B) 4 DAB. IMP density had increased, with some of the larger particles being arranged in short rows (arrows).

C) 4 DAB. The majority of the area of the lateral membrane remained covered with randomly arranged IMPs.

D) 6 DAB. The density of IMPs continued to increase.

Scale bar = 100 nm.
FIGURE 3.50.

IHC lateral membrane maturation.

A) 6 DAB. An increase in the number of large particles arranged in rows was seen (arrows).

B) 10 DAB. The density of both large and small diameter IMPs continued to increase.

c) 16 DAB. The majority of large particles were arranged in rows (arrows), whilst the number of smaller diameter particles had declined.

Scale bar = 100 nm.
FIGURE 3.51.

IHC lateral membrane maturation.

A) 8 DAB. A developing plaque structure composed of tightly packed IMPs.

B) 14 DAB. Small numbers of plaques were present.

C) 16 DAB. The plaques retained an immature appearance.

Scale bar = 100 nm.
FIGURE 3.52.

ZO-1 labelling of the reticular lamina.

A) 2 DAB. ZO-1 is labelled in all the junctions of hair cells and supporting cells in the apical turn.

B) 2 DAB. Middle turn. The regular arrangement of the OHCs is apparent.

C) 2 DAB. Basal turn. Similar intensity of fluorescence was seen in all turns.

Scale bar = 5 μm.
FIGURE 3.53.

ZO-1 labelling of the reticular lamina.

A) **8 DAB.** The expansion of the supporting cell heads is visible.

B) **Adult.** Fluorescence is present in all hair cell and supporting cell junctions.

Scale bar = 10 μm.
FIGURE 3.54.

Schematic diagram of the mature stria vascularis, showing the areas studied. MC = marginal cell; IC = intermediate cell; BC = basal cell; C = capillary; G = gap junction; T = tight junction; M = mitochondria, MT = marginal cell apical tight junction, BG = gap junctions between basal cells and intermediate cells, BT = tight junctions between basal cells.
FIGURE 3.55.

Staining for Na$^+$-K$^+$ ATPase reactivity in the mature stria.

A) Thin section of the mature stria exposed to the complete medium. The presence of Na$^+$-K$^+$ ATPase reactivity is denoted by the black reaction product (open arrow). Staining is seen of the lateral walls (arrows) of the marginal cells (MC) but not on the basal cells (BC).

B) Stria exposed to control solution B, no reactivity is present.

C) Control solution C. No reactivity present.

D) Stria exposed to the complete medium in the presence of ouabain. The inhibitory action of ouabain prevents the activity of the Na$^+$-K$^+$ ATPase enzyme.

Scale bar = 2 $\mu$m
FIGURE 3.56.

The mature stria vascularis.

A) A freeze fracture replica of the apical junctional region of a marginal cell. Junctional strands course both parallel and perpendicularly to the apical surface. Whorl patterns form in the basalmost junctional region (b).

B) A freeze fracture replica of the lateral membrane (LM) of a marginal cell. Densely packed IMPs were present.

Scale bar A = 200 nm; B = 150 nm.
FIGURE 3.57.

The mature stria vascularis.

A) Tight junctional strands seen in freeze fracture replicas of the apical membrane of a basal cell. Numerous junctional strands run in parallel (arrows).

B) Between the tight junctional strands gap junctions form (arrows).

C) Basal cell gap junctions (G) seen in freeze fracture replicas are of regular appearance and are composed of densely packed gap junctional particles.

Scale bar A = 100 nm; B = 125 nm; C = 100 nm.
FIGURE 3.58.

Gross morphological maturation of the stria.

A) 2 DAB. Thin sections of the stria show cuboidal marginal cells (MC) above precursor intermediate cells (IC). Marginal cell apical surfaces have microvilli (arrow) and tight junctions between adjacent marginal cells are already present (open arrow). Pigment granules are present in the precursor intermediate cells.

B) 6 DAB. The process of infolding of the marginal cell lateral membrane has commenced (arrow).

Scale bar = 5 μm.
FIGURE 3.59.

Gross morphological maturation of the stria.

A) 12 DAB. Capillaries (C) have migrated into the body of the stria. Basal cells send processes into the marginal cell layer.

B) 20 DAB. The stria is adult-like in appearance.

Scale bar = 5 μm.
FIGURE 3.60.

Endocochlear potential (EP) trace obtained from a 20 DAB gerbil. EP was approximately 85 mv, the recording being held stable for several minutes.
FIGURE 3.61.

Plot of the onset and maturation of EP recorded from the scala media. EP was first measurable at 10 DAB.
FIGURE 3.62.

Maturation of Na\(^+\)-K\(^+\) ATPase reactivity.

A) 2 DAB. Faint staining for enzyme activity was seen (arrow).

B) 6 DAB. Reactivity was seen on the lateral walls of marginal cells (arrow).

Scale bar = 5 \(\mu m\)
FIGURE 3.63.

Maturation of Na\(^+\)-K\(^+\) ATPase reactivity.

A) **12 DAB.** An almost mature intensity of staining was seen on lateral membranes of marginal cells (arrow).

B) **20 DAB.** Mature staining levels were seen (arrow).

Scale bar = 5 µm.
FIGURE 3.64.

Marginal cell lateral membrane Na\(^+\)-K\(^+\) ATPase reactivity.

A) 6 DAB. Staining is restricted to the infoldings of the marginal cell lateral membrane.

B) 12 DAB. The intensity of staining has increased to almost mature levels.

C) 20 DAB. Mature staining is seen.

Scale bar = 200 nm.
FIGURE 3.65.

Freeze fracture replicas of the marginal cell lateral membrane infoldings.

A) 6 DAB. IMPs are present on the lateral membrane infoldings (arrow).

B) 12 DAB. The IMP density has increased.

C) 20 DAB. The IMP density had reached mature levels.

Scale bar = 200 nm.
FIGURE 3.66.

Maturation of the apical tight junction of marginal cells.

A) 2 DAB. Junctional strands running parallel to the apical surface (a) are present, discontinuous strands form the basalmost junctional region (b).

B) 6 DAB. The basalmost junctional region has increased in complexity, and the apical most region is composed of up to 6 parallel strands.

Scale Bar = 150 nm.
FIGURE 3.67.

Maturation of the apical tight junction of marginal cells.

A) 8 DAB. The basalmost junctional region increased in complexity.

B) 12 DAB. The junction had attained an adult-like form.

Scale bar = 150 nm.
FIGURE 3.68.

Basal cell tight junctional maturation.

A) 6 DAB. Incomplete, broken tight junctional strands first appear (arrow).

B) 8 DAB. The number of junctional strands have increased (arrow).

Scale bar = 100 nm.
FIGURE 3.69.

Basal cell tight junctional maturation.

A) **10 DAB.** The number of junctional strands continue to increase.

B) **12 DAB.** Tight junctional strands arranged in parallel rows are present (arrow).

Scale bar = 100 nm.
FIGURE 3.70.

Basal cell tight junctional maturation.

A) **16 DAB.** Large numbers of densely packed tight junctional strands, running in parallel are present (arrow).

B) **20 DAB.** The tight junctions have an adult-like appearance.

Scale bar = 100 nm
FIGURE 3.71.

Basal cell gap junction maturation.

A) 6 DAB. Apical turn. Small gap junctions appear, widely dispersed on the basal cell membrane.

B) 6 DAB. Basal turn. The gap junctions are larger in size and greater in number than in the apical turn.

Scale bar = 100 nm
FIGURE 3.72.

Basal cell gap junctional maturation.

A) 8 DAB. A greater area of membrane is covered by the developing gap junctions.

B) 12 DAB. The gap junctions continued to expand in area, covering large amounts of the basal cell membrane.

Scale bar = 150 nm.
FIGURE 3.73.

Basal cell gap junctional maturation.

18 DAB. The gap junctional units have reorganised into well defined areas, but still not as regular in appearance as the mature form.

Scale bar = 150 nm.
TABLE 3.1.

Table of measurements of the organ of Corti width and supporting cell dimensions during maturation, taken from the regions shown in Figure 2.1.
### TABLE 3.1
ORGAN OF CORTI AND PILLAR CELL MATURATION.

<table>
<thead>
<tr>
<th>DAB</th>
<th>OC width μm</th>
<th>IP μm</th>
<th>OP μm</th>
<th>D2 μm</th>
<th>D1 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>41.2 (0.26)</td>
<td>49</td>
<td>39.5</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37.2 (2.05)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>38.8 (0.73)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>45  (1.2)</td>
<td>59.2</td>
<td>49.2</td>
<td>5.8</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>42  (0.85)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40.3 (2.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>46.4 (1.4)</td>
<td>61.7</td>
<td>48.7</td>
<td>6.4</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>50.4 (1.45)</td>
<td></td>
<td>50.3</td>
<td>13.8</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>42.9 (1.7)</td>
<td></td>
<td>49</td>
<td>4.2</td>
<td>3.6</td>
</tr>
<tr>
<td>10</td>
<td>59.3 (1.05)</td>
<td>62</td>
<td>52.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>66.5 (1.1)</td>
<td></td>
<td>83.5</td>
<td>15.7</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>51.9 (0.31)</td>
<td></td>
<td>75.4</td>
<td>5.8</td>
<td>5.8</td>
</tr>
<tr>
<td>12</td>
<td>60  (0.75)</td>
<td>86.2</td>
<td>83.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>72.7 (1.2)</td>
<td></td>
<td>92</td>
<td>16.4</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>57.9 (0.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>63  (1.4)</td>
<td>91.8</td>
<td>91.1</td>
<td>20.8</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>84.3 (0.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>64.7 (1.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### PILLAR CELL MEASUREMENTS.

<table>
<thead>
<tr>
<th>DAB</th>
<th>OP-OP DIST. μm</th>
<th>IP-OP DIST. degrees</th>
<th>OP-OP DIST. μm</th>
<th>IP-OP DIST. degrees</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>83.5(0.5)</td>
<td>84.5(2.5)</td>
<td>25.6</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>83.5(1.5)</td>
<td>75(1.2)</td>
<td>44.8</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>78.5(4.5)</td>
<td>70(2.0)</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>62(3.4)</td>
<td>70(0.6)</td>
<td>70.3</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>51.5(0.96)</td>
<td>69.6(2.3)</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>49.6(1.6)</td>
<td>70.8(2.2)</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>AD.</td>
<td>50.7(4.9)</td>
<td>67(2.7)</td>
<td>100.7</td>
<td></td>
</tr>
</tbody>
</table>

*Standard errors given in parentheses.*

268
TABLE 3.2.

Measurements of the basilar membrane dimensions during maturation.
### TABLE 3.2
BASILAR MEMBRANE MATURATION.

<table>
<thead>
<tr>
<th>DAB</th>
<th>Hyaline mass</th>
<th>Tympanic layer</th>
<th>TOTAL</th>
<th>ZA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µm</td>
<td>µm</td>
<td>µm</td>
<td></td>
</tr>
<tr>
<td>2 A</td>
<td>5.05 (0.15)</td>
<td>33.8 (1.1)</td>
<td>69.7 (3.5)</td>
<td>50</td>
</tr>
<tr>
<td>M</td>
<td>4.6 (0.25)</td>
<td>41.3 (0.84)</td>
<td>73.4 (1.4)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>6.2 (0.8)</td>
<td>37.9 (2.6)</td>
<td>63.7 (2.3)</td>
<td></td>
</tr>
<tr>
<td>6 A</td>
<td>17.6 (2.3)</td>
<td>30.7 (0.25)</td>
<td>81.1 (2.9)</td>
<td>46</td>
</tr>
<tr>
<td>M</td>
<td>16.6 (1.3)</td>
<td>44.0 (4.0)</td>
<td>85.3 (5.3)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>15.1 (0.6)</td>
<td>33.9 (1.6)</td>
<td>77.8 (1.6)</td>
<td></td>
</tr>
<tr>
<td>8 A</td>
<td>23.4 (4.1)</td>
<td>21.3 (0.4)</td>
<td>73.1 (4.4)</td>
<td>34</td>
</tr>
<tr>
<td>M</td>
<td>19.8 (0.25)</td>
<td>25.1 (6.6)</td>
<td>84.5 (0.6)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>15.4 (0.8)</td>
<td>35.7 (2.2)</td>
<td>79.4 (1.2)</td>
<td></td>
</tr>
<tr>
<td>10 A</td>
<td>31.7 (1.1)</td>
<td>18.5 (1.7)</td>
<td>68.4 (2.2)</td>
<td>19</td>
</tr>
<tr>
<td>M</td>
<td>27.2 (2.0)</td>
<td>19.6 (0.74)</td>
<td>73.1 (1.12)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>27.6 (1.5)</td>
<td>28.3 (2.8)</td>
<td>81.1 (4.1)</td>
<td></td>
</tr>
<tr>
<td>12 A</td>
<td>24.2 (1.9)</td>
<td>10.5 (1.13)</td>
<td>56.4 (1.6)</td>
<td>13</td>
</tr>
<tr>
<td>M</td>
<td>29.8 (2.9)</td>
<td>14.1 (2.4)</td>
<td>67.5 (3.7)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>23.3 (1.7)</td>
<td>14.9 (2.4)</td>
<td>63.7 (1.8)</td>
<td></td>
</tr>
<tr>
<td>14 A</td>
<td>24.7</td>
<td>6.7</td>
<td>54.7</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>29.9</td>
<td>6.7</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>29.4</td>
<td>12.2</td>
<td>53.8</td>
<td></td>
</tr>
<tr>
<td>AD A</td>
<td>27.2 (1.3)</td>
<td>2.7 (0.4)</td>
<td>44.9 (3.6)</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>31.5 (1.7)</td>
<td>2.9 (0.2)</td>
<td>56.8 (1.87)</td>
<td>9</td>
</tr>
<tr>
<td>B</td>
<td>33.8 (0.75)</td>
<td>3.8 (0.16)</td>
<td>51.6 (2.1)</td>
<td></td>
</tr>
</tbody>
</table>

† Standard errors given in parentheses.
TABLE 3.3.

The maturation of the apical junctional region of the OHC.
TABLE 3.3
OHC APICAL JUNCTION MATURATION.

<table>
<thead>
<tr>
<th>Days after birth</th>
<th>Total junctional depth ( \mu \text{m.} ) ± standard error</th>
<th>number of strands</th>
<th>complexity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.73 (0.02)</td>
<td>4.8 (0.38)</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>0.81 (0.06)</td>
<td>10.1 (1.07)</td>
<td>48</td>
</tr>
<tr>
<td>8</td>
<td>1.04 (0.06)</td>
<td>12.15 (1.7)</td>
<td>73.5</td>
</tr>
<tr>
<td>10</td>
<td>1.39 (0.03)</td>
<td>12.84 (0.51)</td>
<td>77.6</td>
</tr>
<tr>
<td>16</td>
<td>1.65 (0.03)</td>
<td>14.0 (0.48)</td>
<td>97.5</td>
</tr>
<tr>
<td>Adult</td>
<td>1.8 (0.07)</td>
<td>14.7 (0.35)</td>
<td>105</td>
</tr>
</tbody>
</table>

\* Standard errors given in parenthesis.
TABLE 3.4.

Increases in the IMP density and changes in cytoskeletal pillar spacing during maturation of the OHC.
<table>
<thead>
<tr>
<th>DAB</th>
<th>number of IMPs per $\mu m^2$</th>
<th>pillar spacing nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2200 (113.1)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3362 (112.7)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4131 (150.7)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>4275 (164.4)</td>
<td>53.9 (8.2)</td>
</tr>
<tr>
<td>12</td>
<td>4827 (195.3)</td>
<td>44.4 (7.01)</td>
</tr>
<tr>
<td>14</td>
<td>5124 (178.6)</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>5514 (193.4)</td>
<td>38.6 (6.2)</td>
</tr>
<tr>
<td>Adult</td>
<td>5577 (265.8)</td>
<td>35.4 (5.03)</td>
</tr>
</tbody>
</table>

*Standard errors given in parenthesis.*
TABLE 3.5.

Maturation of the IHC apical junctional region.
TABLE 3.5
IHC APICAL JUNCTIONAL MATURATION.

<table>
<thead>
<tr>
<th>Days after birth</th>
<th>Total junctional depth μm.</th>
<th>number of strands</th>
<th>complexity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.56 (0.03)</td>
<td>5.2 (0.2)</td>
<td>36 (5.3)</td>
</tr>
<tr>
<td>4</td>
<td>0.77 (0.02)</td>
<td>9.0 (0.4)</td>
<td>70 (7.9)</td>
</tr>
<tr>
<td>8</td>
<td>0.82 (0.03)</td>
<td>11.7 (0.5)</td>
<td>85 (4.5)</td>
</tr>
<tr>
<td>10</td>
<td>0.99 (0.03)</td>
<td>15.3 (0.6)</td>
<td>109 (17)</td>
</tr>
<tr>
<td>16</td>
<td>1.1 (0.04)</td>
<td>17.3 (0.6)</td>
<td>129 (9.0)</td>
</tr>
<tr>
<td>Adult</td>
<td>1.13 (0.03)</td>
<td>17.4 (0.45)</td>
<td>133 (6.8)</td>
</tr>
</tbody>
</table>

† Standard errors given in parenthesis.
TABLE 3.6.

Changes in the IMP density on the lateral membrane of IHCs during maturation.
TABLE 3.6
IHC LATERAL MEMBRANE MATURATION.

<table>
<thead>
<tr>
<th>Days after birth</th>
<th>number of IMPs per ( \mu m^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>973 (22.3)</td>
</tr>
<tr>
<td>4</td>
<td>1140 (47)</td>
</tr>
<tr>
<td>6</td>
<td>1342 (48.6)</td>
</tr>
<tr>
<td>8</td>
<td>1804 (80.5)</td>
</tr>
<tr>
<td>10</td>
<td>2020 (27.1)</td>
</tr>
<tr>
<td>12</td>
<td>2170 (48)</td>
</tr>
<tr>
<td>16</td>
<td>1375 (36.4)</td>
</tr>
<tr>
<td>Adult</td>
<td>1255 (56.5)</td>
</tr>
</tbody>
</table>

± Standard errors given in parenthesis.
TABLE 3.7.

Maturation of the apical junctional region of the marginal cells of the stria vascularis.
TABLE 3.7
STRIAL MARGINAL CELL APICAL JUNCTION MATURATION.

<table>
<thead>
<tr>
<th>Days after birth</th>
<th>Total junctional depth μm.</th>
<th>number of strands</th>
<th>complexity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.24 (0.04)</td>
<td>6.8 (0.4)</td>
<td>22.3</td>
</tr>
<tr>
<td>6</td>
<td>0.3 (0.09)</td>
<td>10.2 (0.36)</td>
<td>26.5</td>
</tr>
<tr>
<td>8</td>
<td>0.48 (0.07)</td>
<td>12 (0.25)</td>
<td>62</td>
</tr>
<tr>
<td>10</td>
<td>0.5 (0.04)</td>
<td>14.6 (0.36)</td>
<td>88.7</td>
</tr>
<tr>
<td>12</td>
<td>0.73 (0.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0.86 (0.14)</td>
<td>20.7 (0.2)</td>
<td>100</td>
</tr>
<tr>
<td>Adult</td>
<td>0.89 (0.12)</td>
<td>21.0 (0.34)</td>
<td>137</td>
</tr>
</tbody>
</table>

† Standard errors given in parenthesis.
TABLE 3.8.

Maturation of tight junctions on the apical membrane of basal cells of the stria vascularis.
## TABLE 3.8
STRIAL BASAL CELL TIGHT JUNCTION MATURATION.

<table>
<thead>
<tr>
<th>DAB</th>
<th>NUMBER OF JUNCTIONAL STRANDS / μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>34.7 (3.1)</td>
</tr>
<tr>
<td>8</td>
<td>35.4 (7.2)</td>
</tr>
<tr>
<td>10</td>
<td>72.6 (8.7)</td>
</tr>
<tr>
<td>12</td>
<td>118.4 (7.5)</td>
</tr>
<tr>
<td>16</td>
<td>154.3 (5.7)</td>
</tr>
<tr>
<td>Adult</td>
<td>167.5 (10.5)</td>
</tr>
</tbody>
</table>

*Standard errors given in parenthesis.*
TABLE 3.9.

Maturation of the gap junctions on basal cells of the stria vascularis.
### TABLE 3.9
STRIAL BASAL CELL GAP JUNCTION MATURATION.

<table>
<thead>
<tr>
<th>DAB</th>
<th>Mean junctional area $\mu m^2$</th>
<th>Junctional area as % of cell membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.01 (0.01)</td>
<td>6.2</td>
</tr>
<tr>
<td>8</td>
<td>0.02 (0.01)</td>
<td>9.2</td>
</tr>
<tr>
<td>12</td>
<td>0.04 (0.02)</td>
<td>12.7</td>
</tr>
<tr>
<td>16</td>
<td>0.25 (0.08)</td>
<td>26.8</td>
</tr>
<tr>
<td>Adult</td>
<td>0.33 (0.07)</td>
<td>16.9</td>
</tr>
</tbody>
</table>

± Standard errors given in parenthesis.
CHAPTER 4.
DISCUSSION.

4.1 Brief summary of principal findings.
Evidence has been presented for two maturational gradients, a base to apex hair cell gradient and a second gradient beginning in the middle turn, extending to the basal turn and finally the apical turn pertaining to the accessory structures of the organ of Corti. The maturation of supporting cells was found to proceed in two main stages. Prior to 8 DAB, extension of the Deiters' cell heads contributed to the increase in width of the organ of Corti. Subsequently, enlargement of the inner pillar cell heads accounted for the majority of the increasing dimensions. The major morphological event, the opening of the spaces between the pillar cells and around the OHCs, occurred after 8 DAB, commencing in the middle turn. The basilar membrane at 2 DAB consisted of a thin hyaline layer between the organ of Corti supporting cells and a thick epithelial cell layer on the tympanic side. Between 2 and 20 DAB the hyaline layer increased in thickness whilst the tympanic layer thinned to a single cell. This delayed maturation of the basilar membrane would have important consequences for the mechanical response of the organ of Corti, and may be reflected in the maturation of cochlear responses.

Outer hair cells were found to mature before the inner hair cells, with the exception of the junctional region around the neck of the cells. This was more complex and reached a mature configuration in IHCs before that of the OHCs. A sequential pattern of maturation of the lateral membranes of IHC and OHCs was found.
In OHCs, the number of IMPs, thought to be the sites of OHC motor elements, increased from 2 DAB onward, reaching a mature density by 16 DAB. The first evidence of the sub-surface cisternal network of the OHC did not appear until 8 DAB, whilst evidence of the pillar structures thought to couple the cisternal network to the plasma membrane was not seen until 10 DAB. Since motility can be elicited from 8 DAB onward in OHCs (He et al 1994), this suggests that the IMPs on the lateral wall of the OHC are the sites of the putative OHC motor elements rather than the cisternal network.

IHC lateral membranes also exhibited a complex sequential pattern of IMP maturation. Between 2 and 10 DAB, large numbers of small IMPs were distributed over the lateral membrane. From 10 DAB onward the numbers of these small particles declined. Larger IMPs were also present at 2 DAB, apparently randomly distributed about the membrane. By 6 DAB these had begun to organise in rows, which by 16 DAB were present in large numbers. Plaque aggregations of IMPs did not appear on the lateral membrane until 8 DAB and had not attained adult-like numbers by 16 DAB. The CM was first recordable at 10 DAB, the CAP at 12 DAB and the SFOAE at 14 DAB. Therefore, the maturation of the IHC lateral membrane continued after the onset of auditory function, and proceeded throughout the period of increasing functional response magnitude and sensitivity.

The study of the stria vascularis showed the marginal cells to have mature apical tight junctions by 12 DAB, by which time the activity of the Na⁺-K⁺ ATPase enzyme had also attained adult-like levels. The EP was first recordable from 10 DAB onward, rising steeply between 12- 20 DAB. It was found that the period of EP onset and rise coincided
with the appearance and maturation of tight and gap junctions of the basal cells, suggesting a major role for these membrane specialisations in the onset and generation of EP.

4.2. Gross morphological maturation.

The changes in the gross anatomy of the gerbil organ of Corti during the first three weeks post natally described here are in general agreement with studies of the maturation of other rodent hearing organs. The timing of maturational events is delayed by 2-4 days compared to the rat or mouse and the gradient of maturation for the sensory cells conforms to the base to apex gradient described in other species. On the evidence of apical surface features, IHCs appear to mature after the OHCs, similar to rat (Lenoir et al 1987) but possibly different from the mouse (Lim and Anniko 1985). The results presented in this study show that the maturation of the accessory structures, the pillar cells and basilar membrane, is not complete until 20 DAB, with substantial changes occurring coincident with the onset and development of acoustically evoked cochlear responses. The maturation of the tectorial membrane proceeds in a similar manner to the described by Lim (1977) in the cat and Lenoir et al (1987) in the rat.

A. Opening of spaces.

At 2 DAB the organ of Corti comprises tightly packed hair cells and supporting cells, which by 16 DAB had attained the appearance substantially that of the adult. The most radical of the changes on the gross level, the opening of the tunnel of Corti and the spaces of Nuel around the OHCs, is first evident between 8 and 10 DAB. Ito et al (1995) noted that opening of the tunnel of Corti commenced as early as 4 DAB in the gerbil, however there was no evidence seen in this study of separation occurring at the 4 DAB stage.
The development of the spaces begins with an apparent loosening of the contacts between the OHCs and their adjacent supporting cells which occurs from 8 DAB onward. Separation of the inner and outer pillar cells appears to occur slightly later than this. The appearance of the spaces may be related to changes in the expression of cell adhesion molecules. Changes in the expression of the Ca\textsuperscript{2+} dependent cell adhesion molecule E-cadherin during maturation of the mouse organ of Corti have been described by Whitlon (1993). The molecule was found to disappear from the lateral walls of those cells which would be exposed to fluid spaces, this down regulation occurring coincident with the appearance of the fluid spaces. The role of cell adhesion molecules is discussed in more detail below.

Inner and outer pillar cells become separated along their lateral wall beginning around 8 DAB, causing the tunnel of Corti to open. Prior to the appearance of the spaces, the IP and OPs increase in size, whilst remaining in close contact. By 10 DAB the separation of the IP and OPs had begun, initially at the bases of the IP and OP cells, spreading upward toward the reticular lamina. The bases of the OPs move outward to a position over a "hinge" formed by the thin part of the basilar membrane between the zona pectinata and zona arcuata, reaching their final position after 12 DAB. The bases of the inner pillar cell drift inward a shorter distance toward the spiral lamina. The tunnel of Corti expands due to this apparent movement of the bases of the pillar cells. It is unclear whether the OPs "slide" over the basilar membrane, extending the length of their bases, or the basilar membrane itself increases in width taking the OPs with it. Harris et al (1990) reported that the length of the modiolus, hence basilar membrane length, had
reached adult values before the onset of physiological responses at 12 DAB. However, the results presented in this thesis show that changes continue to occur in basilar membrane width, and organ of Corti width, beyond this period. This is evident from changes in the distance between the extreme edges of the IP and OP cells and the width of the zona arcuata between 10-20 DAB. This suggests that the zona arcuata expands perhaps carrying the OP bases with it. In addition, the expansion of the IP heads which occurs over the same time period may also act to push the cells apart.

Increases in the size of the heads of the IPs begin at 8 DAB, whilst Kraus and Albach-Kraus (1981) reported that enlargement of the heads of the pillar cells in the mouse occurred between 5 and 10 DAB. Prior to 8 DAB, the increase in the width of the organ of Corti is due to increases in the size of the heads of the Deiters' cells. Both the increase in Deiters' cell heads seen between 6-8 DAB and the increase in the IP heads between 8-12 DAB occurred first in the middle turn. The IP cell heads continue to enlarge between 12-20 DAB, coincident with the increase in width of the zona arcuata. Since this period coincides with the onset of recordable auditory function and the establishment of fine tuning as described in this study and others (for example Woolf and Ryan 1984, McGuirt et al 1995), it may be concluded that the maturation of these supporting structures will continue to influence the mechanical properties of the organ of Corti throughout this period (12-20 DAB), and may be relevant to the changing tonotopicity seen in the developing gerbil cochlea, which is discussed below.

B. Basilar membrane.

In other mammals, the basilar membrane tends to be thicker in the basal region. However, in gerbils the basilar membrane is thickest in the middle turn (Plassmann et al...
M. unguiculatis, used in this study, is one of the gerbil species having, in the adult, a large hyaline mass underneath the zona pectinata of basilar membrane and a much thinner layer below the zona arcuata. This morphological structure is not found in rodents such as rats and mice (Lay 1972), although a similar structure is found in the kangaroo rat (Webster and Webster 1977) and some species of bat (Kossl and Vater 1985a, Dannhof and Bruns 1991). Those gerbil species (e.g., P. obesus) with the largest hyaline mass have the best sensitivities to low and mid frequencies (Plassmann et al. 1987). M. unguiculatis has a wide range of best sensitivity between 0.8 and 16 kHz (Ryan 1976), and has the hyaline mass present in all three turns. Since the Hensen’s cells are hypertrophied in the mature gerbil organ of Corti, the hyaline structure would serve to contribute added mass to the organ of Corti. This would increase damping which in turn may increase the low frequency response of the system, in effect extending the amount of basilar membrane responsive to lower frequencies (Lay 1972, Brown 1987).

Gerbils are known to have particularly strong OAEs (Brown 1987), as has the mustache bat, which also has a thickened basilar membrane in behaviourally important frequency regions (Kossl and Vater 1985b). Kossl and Vater (1985a) speculated that vibration of the basilar membrane would be enhanced apically where there was no thickening and this would contribute to sensitivity and the production of large OAEs. Using the ratio of thickness/width as an estimate of basilar membrane stiffness, employing data from this study for the basilar membrane thickness and data from Plassmann et al. (1987, Figure 4) for basilar membrane width, the values for basilar membrane stiffness were calculated to be 0.16 for the apical turn; 0.24 for the middle turn; and 0.15 for the basal turn. These data suggest that the basilar membrane is stiffest in the middle turn, with the apical
and basal turns being of approximately the same stiffness. As with similar measures of basilar membrane stiffness from the mustache bat (Kossel and Vater 1985b), this "reverse" stiffness does not appear to be reflected in frequency mapping in the adult gerbil. One may speculate that the presence of active mechanics, driven by the OHCs, masks the stiffness effect in the adult, and one contribution to the changing tonotopicity of the developing gerbil cochlea may be from changing basilar membrane stiffness as determined by changes in its thickness as maturation progresses and as the active OHC elements are coupled more efficiently to the basilar membrane vibration. In effect, the immature gerbil organ of Corti would only be responsive to lower frequency stimuli due to the increased loading of the hyaline/tympanic epithelial cell layer structure, particularly in the basal turn, and the presence of "passive" micromechanics.

In this study, thickening of the hyaline mass was found to occur in the middle turn before the basal turn and reached mature values in all turns by 20 DAB. The final dimensions of the basilar membrane were attained by a combination of increasing hyaline mass thickening and a decrease in the tympanic epithelial layer. Echteler (1994) found the structure of the gerbil organ of Corti in the middle turn to be more mature at 10-13 DAB, the basilar membrane in the middle turn reaching adult thickness at 12 DAB and continuing to decrease in the base until about 15 DAB. The basal turn basilar membrane did not reach mature thickness until around 20 DAB in this study, somewhat later than Echtelers' (1994) findings.

It has been reported that the gerbil basilar membrane ceases longitudinal growth by 10 DAB (Harris et al 1990), suggesting that the changes in frequency sensitivity and
selectivity seen after the onset of auditory function at 12 DAB (discussed below) were not due to increasing dimensions of the basilar membrane. In this study, substantial changes in the basilar membrane thickness and increases in the width of the zona arcuata region occur in the period 10-20 DAB. These changes, increasing thickness of the hyaline mass and decreasing tympanic cover layer, are coincident with the time course of CAP maturation and the development of fine tuning. Together with the changes in the supporting cell dimensions noted above, the findings of this study suggest that substantial changes occur in the mechanical properties of the organ of Corti between 12-20 DAB, and these are probably reflected in the rate of maturation of acoustic stimulus-evoked cochlear responses.

C. Maturation of cochlear responses.

The gerbil differs from most other mammalian species in that the middle ear conductive apparatus matures before the onset of physiological responses (Finck et al 1972), although there is more recent evidence that middle ear conduction continues to improve between 10-19 DAB (Cohen et al 1991). The first recordable CAP responses to auditory stimuli from the gerbil cochlea were obtained at 12 DAB in this study, although direct mechanical stimulation of the stapes elicits responses at 10 DAB (Woolf and Ryan 1988). Sound evoked CMs were first recorded at 10 DAB in this study, and are almost mature by 18 DAB, although evidence from this study and McGuirt et al (1995) suggests that CM continues to increase beyond 20 DAB. The onset and increase of the endocochlear potential may play a role in the development of the cochlear potentials, and has been found to increase rapidly after 10 DAB, as described in this study and by other workers (Woolf and Ryan 1988, McGuirt et al 1995).
Paradoxically, responses to low frequency stimuli are first to appear, whilst anatomical studies had suggested that the basal turn of the cochlea, which in the adult responds preferentially to high frequency stimuli, matured first. There is now much evidence for changes in the tonotopic organisation of the developing gerbil cochlea, for example increasing characteristic frequency of the CM (Arjmand et al 1988), summating potential (Yancey and Dallos 1985), increasing characteristic frequency of spiral ganglion cells (Echteler et al 1989) and shifts to higher frequencies of the responses of more central auditory nuclei (Sanes and Rubel 1988, Sanes et al 1989).

According to one hypothesis to account for this discrepancy, Rubel (1978) suggested that the basal region was the first to transduce vibrations of the partition into neural signals, reflecting the base to apex maturation gradient, but the increased mass due to immature structures could damp the basilar membrane motion restricting it to low frequencies. As development progressed, with loss of mass, the vibration would increase in frequency. An alternative hypothesis based on differential maturation of the "tail" and "tip" of the tuning curve was proposed by Romand (1987). Since the OHCs are generally accepted as being necessary to produce the "tip" of the tuning curve, it was suggested that the low frequency responses which appeared first originated from mainly IHC activity. The appearance of the high frequency responses coincided with the onset of active OHC responses and their contribution to the micromechanical response of the organ of Corti.

Otoacoustic emissions are thought to have their origins in nonlinear micromechanical properties which underlie the frequency selectivity and sensitivity of the cochlea, particularly OHC motility. SFOAEs are thought to originate from a restricted region of
the organ of Corti which responds maximally to the acoustic stimulus frequency. Therefore, SFOAEs may be considered as reflecting the active, tuned mechanical response of the organ of Corti. The results of this study show that the tuned micromechanical response appears after the CM but coincident with the CAP, which is produced by activity at the IHC-afferent nerve synapse. The maturation of the SFOAE responses described in this study show a similar time course as the CAP, both of these response measures lagging the CM by several days. This suggests that the OHCs are functional by 12 DAB but the necessary stimulus coupling of the OHC to the IHC remains immature. This would be expected from the lack of structural maturity seen at 12 DAB. Norton et al (1991) studied the development of the 2f1-f2 DP in the gerbil. Their results agreed with those from rat (Lenoir and Puel 1987, Henley et al 1989) in that responses to high frequencies appeared first at 13-14 DAB with low frequency responses appearing last at 18-19 DAB. Norton et al (1991) suggested that the development of the active mechanism of the OHCs, and consequently a change from "passive" to "active" modes of vibration of the organ of Corti, as reflected in the appearance of DPs, underlies the development of the shifts in the place code map. However, He et al (1994), in their study of the onset of OHC motility, found that motility could be elicited from 8 DAB onward and that OHCs from all turns exhibited motility by 12 DAB. Thus, OHCs are capable of motility before the onset of measurable, acoustically evoked cochlear responses. The results of the study, discussed below, of OHC lateral membrane maturation show the onset of motility coincides with increasing protein particle density in the lateral membrane, suggesting the OHC motor elements were in place, this being followed by the organisation of the cisternae and the pillar structures associated with cortical lattice.
The data suggest that it is not the OHCs' capability of motility per se which is the limiting factor of cochlear response maturation, rather immaturity in the coupling of the OHC-tectorial membrane-basilar membrane complex which constrains the cochlear responses to acoustic stimuli between 10-20 DAB. Changes throughout this period in the basilar membrane thickness and pillar cell morphologies probably contribute to the changing tonotopicity of the gerbil cochlea and the delay between the onset of OHC motility and mature frequency response characteristics.

4.3 OHC maturation.

A. The apical membrane.

The apical surface of the gerbil OHC exhibited a base to apex maturation gradient, similar to that reported in other species (Lenoir et al 1987, Roth and Bruns 1992b). Basal turn OHCs lost their extranumary microvilli first, commencing at 6 DAB, with several days lag between OHCs in the basal turn and the loss of microvilli on the OHCs from the apex. The excess microvilli on the inside of the stereociliary rows of all turns had reabsorbed by 12 DAB. The kinocilia were also reabsorbed by 12 DAB, although some freeze fracture replicas showed remnants of kinocilia up to 16 DAB, particularly in the apical turns.

Apical membranes of the immature hair cells showed the presence of "pits" in freeze fracture replicas. These may be related to coated vesicles which are seen to fuse with the membrane covering regions where the cuticular plate is immature. Forge and Richardson (1993) have previously shown these vesicles to be present in the hair cells of organotypic cultures of immature mouse organ of Corti. The presence of these vesicles in gerbils in vivo shows they are not an artifact of in vitro conditions. At 2 DAB there were large
numbers of vesicle sites, some of which occurred between the microvilli. These "pits" decreased in number until by 8 DAB they were limited to the lateral edges of the cell. This suggests that large numbers of coated vesicles may be a feature of immature hair cells.

In the mature guinea pig, the OHC cuticular plate extends to meet the junctional complex. Gaps in the cuticular plate around the lateral edge of the cuticular plate have been observed and in the region of the basal body of the kinocilium (Steyger et al 1989). Thorne et al (1987) reported the absence of phalloidin staining of actin in the cuticular plate-free region and other locations around the lateral margin of the OHCs. Kachar et al (1993) noted the presence of vesicles in the cuticular plate gap in bullfrog macular sacculae and suggested these actin free regions served as routes of vesicular transport. Densely packed actin in the cuticular plate is likely to impede the access of vesicles to the membrane. The presence of pits over large areas of the surface of the cuticular plate region between 2 and 8 DAB suggests that the passage of vesicles to the apical membrane is less restricted than in the mature OHC, possibly due to actin being less densely packed between these ages than in the adult, as well as being indicative of a high rate of membrane turnover. There was evidence of rows of vesicles which appeared to be associated with microtubules which extended down from the kinocilium basal body, and it has been suggested that these microtubules may provide a transport route for the vesicles to the apical surface of the cell (Kachar et al 1993). At 2 DAB the cuticular plate was less extensive, with a meshwork of microtubules extending laterally. At 8 DAB, the cuticular plate was denser staining, extending almost to the edge of the cell, but there remained some regions, particularly at the cell edges, with concentrations of vesicles. By
10 DAB, the cuticular plate extended almost to its mature size, with the denser staining junctional region being well defined. Weaver et al (1994) noted that actin labelling of the cuticular plate increased from 10 DAB (their starting point) until mature labelling was seen at 16 DAB. This agrees with the findings in this study of the maturation of the cuticular plate and the progressive limiting of vesicles to the outer edges of the OHC apical surface.

B. The junctional region.

The junctional area at the apical end of the cell may be considered as comprising two regions, defined by the orientation of the strands composing the junction (Jahnke 1975, Gulley and Reese 1976, Iurato et al 1976, Nadol 1978). In the mature OHC, the apicalmost junctional region comprises up to 6 strands running parallel to the apical surface, occupying around 15% of the total junctional depth. Basal to this is a region of junctional strands running perpendicular or at angles to the apical surface, enclosing roughly polygonal-shaped areas. Both these regions are identifiable at 2 DAB. The former region has the characteristics of a tight occluding junction maintaining a permeability barrier (Claude and Goodenough 1973). The tight junction associated protein ZO-1 has been found at all junctions of the reticular lamina at 2 DAB onward in this study, and also at high levels in the guinea pig junctional region (Kachar 1995). Although the total junctional depth continued to increase until 16 DAB, there did not appear to be any great increase in the depth of the labelling of the ZO-1. This may suggest that although the strands forming the basalmost junctional region appear similar to those of the tight junction region, ZO-1 may not be localised to the membrane in this region, and therefore the more complex basalmost region may have an alternative function, for example as a specialised form of adherens junction.
As an adherens junction, the basalmost region may have a role in mechanically coupling the OHCs and supporting cells. Adherens junctions are connection sites for actin filaments, and in epithelial cells form bands around adjacent cells just below the tight junctional region. The presence of filaments seen in thin sections on the cytoplasmic side of both supporting cells and OHCs only in the region of the basal junctional area, as well as the observation that the actin rich cuticular plate extends to only the basal junctional region, may suggest an adherens role for this area. However, since adherens junctions do not usually show a distinct morphology in freeze fracture replicas, if the basal area is such a junction it would be of an unusual type.

At 2 DAB, the apical junctional region was present as a single continuous strand, together with an incomplete second strand. This region had reached a mature configuration by 8 DAB. The basal junctional region at 2 DAB consisted mainly of strands formed into a few polygonal regions which extended to a depth of around 40% of the mature junctional size. This region reached maturity later than the apicalmost region, the number of tight junctional strands reaching an almost adult number by 12 DAB, with slight increases in number until the adult form was reached by 16 DAB. The complexity of the junction can be quantified in terms of the number of branching points per unit area, and this was found to reach adult levels by 16 DAB.

The differing maturity rates may be interpreted in relation to possible functional roles of the two junctional regions. In order to maintain the large endocochlear potential (EP) which exists across the apical surface of the reticular lamina, an efficient seal must be created between the constituent cells of the reticular lamina. The apical region of parallel
strands is a candidate for this sealing function, this configuration of strands having been related to junctional permeability in many systems, eg airways, in which decreasing permeability was correlated with an increasing number of parallel strands (Schneeberger and McCormack 1984). In the maturing gerbil organ of Corti, the establishment of an effective reticular lamina seal would be essential for the establishment and maintenance of the EP. EP was first measurable at 10 DAB in this study, by which stage the apical tight junctional region has reached a mature configuration. This would allow EP to rapidly build up with minimal leakage. Further evidence for a sealing role for this apical junctional region is that apical junctions between Hensens cells, which also form part of the reticular lamina, have only parallel oriented strands (McDowell et al 1989), indicating that this region of the OHC membrane is of primary importance to maintaining the permeability barrier. The uniformity of labelling for ZO-1 across the reticular lamina, from the sulcus to the Hensens cells, supports this interpretation.

Maturation of the basal junctional region took place over a longer period. Whilst the apical region was mature around 8 DAB, the basal region at this stage remained less complex and less extensive than in the adult. This region has previously been noted as apposing the cytoskeletal elements in the adjacent supporting cell (McDowell et al 1989). The increasing complexity of the basal junctional region occurs coincident with the appearance of darkly staining material in the adjacent supporting cells and the realignment of the microtubule bundles of the pillar and Deiters' cells (Henderson et al 1995). These authors termed the concentration of specialised cytoskeletal material and junctions "surfoskelesomes" in the supporting cells. In the developing gerbil OHC, the junctional region can be seen as a darkly staining area with associated fibrillar tufts on the
cytoplasmic side before the cuticular plate has extended to the junction border. From 10 DAB onward, the fluid spaces of the organ of Corti begin to open, with the mature configuration of the basal junctional region developing as the rearrangement of the organ of Corti supporting structures occurs. If the basal junctional region performs a structural, stabilising role attaching the OHCs to the surrounding supporting cells (Jahnke 1975), it would be logical for the junction to form as the supporting cell configuration matured, and junction formation may be facilitated by the same factors which trigger the fluid space opening. As noted above, the intensity of labelling for the tight junctional protein ZO-1 did not substantially increase from 2 DAB onward. This may suggest that ZO-1 is absent from the basalmost region, implying the involvement of other cell adhesion molecules in the assembly of the junction in this region. Possible candidates for the facilitation role in the opening of the spaces around the OHCs and in the formation of the basalmost junctional region may be one or more of the cadherin family. The role of cell adhesion molecules and junctional proteins in the maturation of the OHC- supporting cell junctions are discussed in more detail below.

C. Lateral membrane maturation and OHC electromotility.

Cell shape in the OHC is thought to be maintained by the cortical lattice, a cytoskeleton composed of actin and spectrin which lies beneath the plasma membrane (Holley and Ashmore 1988, 1990). Force generation linked to OHC motility is believed to originate in the plasma membrane (Holley et al 1991, Kalinec et al 1992), with the pillars, which link the plasma membrane to the cytoskeletal filaments, coupling the force generation to cell deformation. The high protein content of the OHC plasma membrane (Forge 1991) may have a structural role in supporting the cytoskeletal lattice (Holley et al 1992), with some of the membrane particles seen in freeze fracture replicas of OHC lateral
membranes possibly also being the OHC motor elements (Kalinec et al 1992). The IMPs may, however, also represent other features, such as membrane ion channels for calcium and potassium. In this study, evidence is presented that the components of this plasma membrane-cytoskeletal complex mature at differing rates in the gerbil OHC, suggesting differing roles in the motility mechanism.

i) Lateral membrane particles.

Freeze fracture replicas show that IMPs are present at 2 DAB, the numbers increasing from means of 2200 per $\mu m^2$ at 2 DAB to 3362 per $\mu m^2$ by 6 DAB in middle turn OHCs (Table II). Basal turn OHC electromotility is first elicited at 7 DAB in the gerbil and at 8 DAB in apical turn OHCs (He et al 1994). In this study a mean of 4131 particles per $\mu m^2$ at 8 DAB was found. These figures represent the total IMP count, since although a population of smaller particles was also present and clearly identifiable between 2 to 6 DAB, it was not possible to distinguish the large and small particle populations after 6 DAB due to the high density of the IMP packing. Consequently, counts of the total IMP particle density were made from middle turn OHCs, although it appeared that it was predominantly the large particles which were increasing in number. The IMP counts obtained suggest that, if the large particles are the motor elements as proposed by Kalinec et al (1992), a figure of around 4000 IMP per $\mu m^2$ is required as the threshold for motility in so far as number of motor elements is concerned. However, structural factors thought to couple the motors to the cell cytoskeleton appear to mature after 8 DAB, and these are discussed below.

100% of gerbil OHCs exhibit electromotility by 12 DAB (He et al 1994), when the onset of auditory function was recorded in this study. The results of the IMP count show they
had reached a mean density of 4827 per $\mu\text{m}^2$ by 12 DAB, almost 87% of the adult density. He et al (1994) presented evidence that by 14 DAB mature OHC motility response amplitudes were reached. At this stage we found the IMP count to be a mean of 5124 per $\mu\text{m}^2$, approaching 92% of the adult number of about 5577 per $\mu\text{m}^2$ in the adult. These data imply that OHC motile capability is more closely related to IMP density than cisternal layer number, which did not reach adult values until 16 DAB.

As noted previously, not all IMPs may, however, be putative motor elements. Some may represent ion channels whilst others may have a structural role in maintaining cortical tension (Holley et al 1992). Evidence from this study suggests that the OHC lacks rigidity as the fluid spaces open, at 10 DAB, but appears more rigid by 12 DAB. As well as being in accord with increasing IMP density and increasing numbers of cisternal layers seen during this period, the number of cytoskeletal pillars also increases markedly during this period. However, since basal turn OHCs possess only a single cisternal layer, the increasing rigidity may be more related to the progressive maturation of the cortical lattice complex with which the pillars are associated.
ii) Sub-surface cisternae.

Prior to 8 DAB, no evidence of cisternal structures were seen. By 8 DAB, broken lengths of cisternae had formed extending down the lateral membrane almost to the level of the nucleus. By 12 DAB in the middle turn, 2-3 cisternal layers had developed and an adult complement of 5 layers in middle turn OHCs was reached by 16 DAB. The post 10 DAB findings of progressively increasing cisternal layer numbers in the middle turn are in agreement with those reported by Weaver et al (1994).

Pujol et al (1991) found that the appearance of the first layer of cisternae coincided with the onset of motility in foetal guinea pig OHCs. In the gerbil OHC, Weaver et al (1994) have shown that at 10 DAB a single cisternal layer is present. This study has shown that this layer was present at 8 DAB, albeit in isolated lengths in some samples, just after the onset of motility of gerbil OHCs reported by He et al (1994). The lack of a layer of cisternae extending from just below the junctional region to the level of the nucleus in some 8 DAB OHCs may underlie the observation of He et al (1994) that only between 20-50% of OHC exhibit motility at 8 DAB. There does not appear to be a simple relationship between the number of cisternal layers and OHC motility (Pujol et al 1991), since OHCs in several species are known to have only a single layer of cisternae, for example those of the horseshoe bat (Vater et al 1992), human (Arnold and Anniko 1989), guinea pig (Bannister et al 1988, Forge et al 1993b) and mouse (Forge 1991), as well as those from the basal turn of the gerbil. The organization of the first cisternal layer may be the critical factor in determining motility (Holley and Ashmore 1990) and the appearance of the extra cisternal layers after 10 DAB may be related to Ca^{2+} storage and release (Flock et al 1986) or a specialisation related to the natural habitat and acoustic
iii) Cytoskeletal pillars.

The pillars connecting the outermost cisternal layer to the plasma membrane were first discernable at 10 DAB. No evidence of pillar structures in OHCs prior to 10 DAB was found in this study. At 10 DAB the pillars were present in small numbers, being irregularly spaced. Larger numbers were present by 12 DAB and had reached almost mature numbers by 16 DAB. The spacing of the pillars decreased from means of 53.9 nm at 10 DAB to 38.6 nm by 16 DAB. However, a lack of optimal staining conditions for the pillar structures may be responsible for them being undetected prior to 8 DAB in this study and possibly the use of deep etching after freeze fracture may resolve this question. These pillars are thought to be part of the cortical cytoskeletal lattice of OHCs (Bannister et al 1988), which is thought to be composed of actin and spectrin (Holley and Ashmore 1990). From immunofluorescence studies, actin is known to be associated with the mature cortical cytoskeleton (Holley and Ashmore 1990). In the developing gerbil OHC, Weaver et al (1994) have shown that at 10 DAB there was little actin labelling next to the lateral membrane. The labelling increased significantly by 12/13 DAB. This would agree with the findings of this study, which show that at 10 DAB the pillar structures were much fewer in number and more widely spaced than in the mature OHC. The number and density of pillars had increased by 12 DAB, with an interpillar distance of 44.4 nm (80% of the mature spacing). At this stage He et al (1994) elicited motility in 100% of OHCs studied, with adult response amplitudes being seen at 14 DAB. In this study the number of pillars continues to increase after 12 DAB, so it may be suggested that the pillars are probably involved in increasing the efficiency of the coupling of the motor elements in the plasma membrane to the cortical cytoskeleton (Holley et al 1992),
rather than being the site of force generation (Bannister et al 1988).

D. Basal Membrane.

Synaptogenesis has been studied in a variety of species, including human (Lavigne-Rebillard and Pujol 1990), cat (Pujol et al 1978), mouse (Kikuchi and Hilding 1965), rat (Lenoir et al 1980), and gerbil (Echteler 1992). A characteristic sequence of synaptogenesis has been observed by the above authors, with OHCs being initially contacted only by afferent fibres and their characteristic efferent innervation being seen at a later stage (Lenoir et al 1980, Roth and Bruns 1993). In the gerbil, Echteler (1992) has described the developmental segregation of the OHC and IHC populations of afferent fibres, with OHC and IHC afferent populations becoming segregated by 6 DAB.

The results of this study show that at 2 DAB afferent fibres were present beneath OHCs, freeze fracture replicas of the basal membranes having evidence of pits on the membrane surface which are the sites of vesicle exocytosis from the OHC cell body. Thin sections showed small regions of membrane thickening, possibly associated with these active synaptic regions. By 6 DAB, afferent fibres cover the OHC basal membrane, with an increased number of vesicles. The fibres ran along the OHC rows, with little cross-row branching and no evidence of OHC-IHC branching. This is in agreement with Echtelers' (1992) findings regarding the timing of segregation of the IHC-OHC afferent populations. No evidence of direct efferent synapses on OHCs before 12 DAB was found in this study, when the existing afferent fibres appear to decrease the size of their synaptic contacts. The presence of increasing numbers of vesicles between 2 and 10 DAB together with membrane features typical of afferent synapses during this period suggests the
synapses are functional, although their precise role is unclear.

E. Summary I.

This section of the thesis has described the maturation sequence of various membrane regions of the gerbil OHC. The apical surface is mature in appearance by 12 DAB, but some apical OHC retain immature characteristics, for example endocytotic vesicle openings and traces of kinocilium, until 16 DAB. The apicalmost part of the junction around the neck of the OHC exhibits adult-like characteristics by 8 DAB, whilst the basal junction region is present in simple form at 2 DAB and continues to increase in complexity until it reaches a mature state by 16 DAB. Also present at 2 DAB are intramembrane protein particles. These are the putative motor elements of the OHCs, and a mature density is attained about 16 DAB, the density having increased from a mean of 2200 to 5577 per μm² at 16 DAB. Middle turn OHC had approximately 75% of their mature IMP count at the time of electromotility onset given by He et al (1994), rising to 92% when they found mature motile response amplitudes at 14 DAB. At 8 DAB subsurface cisternae were first seen, the first row being complete by 10 DAB, at which stage the cytoskeletal pillars first became apparent.

The minimum conditions for functional OHCs may comprise the number of motor elements (IMPs) and the presence of a means of coupling the force to the membrane (the pillars). Maturational processes occurring in other areas of the cochlea obviously contribute to the total in vivo picture. For example, at the gross anatomical level, this study shows that the organ of Corti accessory structures, the supporting cells and basilar membrane, did not reach maturity until 20 DAB. In addition, the acoustic stimulus-
evoked potentials recorded in this study, the CM and CAP, continued to increase between 12 and 20 DAB. This is in accord with Echteler et al (1989) who showed that the neural responses of spiral ganglion cells in gerbil become more sharply tuned between 14 and 17 DAB. This period, 12-20 DAB, also coincides with the time that the middle ear becomes fully mature and clear of debris (Woolf and Ryan 1988). These factors would impose constraints upon the organ of Corti response to acoustic stimulation, indeed measures of cochlear performance are known to continue to mature up to several weeks after the initial onset of hearing, for example distortion product amplitude (Norton et al 1991).

The maturation of the gerbil OHCs can therefore be summarised as comprising three stages. An initial postnatal phase between 2-8 DAB, when the OHC is still surrounded by supporting cells with no extracellular spaces, which encompasses the initial period of apical junction formation and a rapid increase in lateral membrane IMP density. This is followed by an intermediate period at 8 to 10 DAB characterised by the beginning of space opening and the first appearance of subplasmallemal specialisations of lateral wall of the OHC. Between 8 and 10 DAB, the rate of IMP inclusion into the lateral membrane substantially decreases. A final period between 12 and 16 DAB coincides with the maturation of the electromotile response and the development of fine tuning, accompanied by a final increase in IMP density to attain adult values and the maturation of the basalmost region of the cell-neck junctional region. Maturation of the supporting cells and basilar membrane also occur up to 20 DAB, suggesting that the mechanical response of the organ of Corti and the coupling of OHC motility to basilar membrane motion occur coincident with the increasing stimulus-evoked cochlear responses.
4.4 IHC maturation.

The principal finding of this section of the thesis is that maturation of the lateral wall of the IHC continues beyond the onset of auditory function and during the period of increasing cochlear response sensitivity.

To date, only morphological studies using SEM and thin sections have been carried out describing IHC maturation (for example Lenoir et al 1980, Lim and Anniko 1985, Roth and Bruns 1992b, Kaltenbach and Falzarano 1994). This study provides quantitative data on the junctional and lateral membrane maturation of the IHC. There are no direct measurements of IHC intracellular potential changes during maturation in vivo available, however Kros et al (1991) have studied the maturation of the K⁺ current through IHCs from mouse organ of Corti in vitro. They found an increase in the magnitude of the outward K⁺ current to occur coincident with the onset of hearing in vivo (Kros et al 1991).

A. The apical membrane.

The data presented in this thesis shows that the apical and lateral membrane of IHCs mature after those of the OHCs in the gerbil organ of Corti. Although the IHC apical membrane does not attain an adult-like appearance until after that of the OHCs, at 2 DAB the IHC stereocilia are thicker and more well defined than those of the OHCs. This is in agreement with Lenoir et al (1987), who found that the stereocilia first appeared on IHCs in the rat organ of Corti. Romand et al (1993) found actin to appear first in the IHCs of rat pups at 18 days of gestation, before its appearance in OHCs. The finding that fimbrin,
which acts as an actin cross-linking protein, is expressed in IHC stereocilia before those of OHCs in the developing rat organ of Corti (Zine et al 1995), further illustrates the paradoxical finding of more mature stereocilia on IHCs whilst the presence microvilli on the apical surface would suggest that IHCs mature after the OHCs. In addition, the junctional region around the neck of the IHC appears to be more mature at 2 DAB than that of the OHC.

Kaltenbach and Falzarano (1994) found that the IHC from the hamster organ of Corti were morphologically mature before the onset of hearing, and that the maturation of apical turn IHCs lags that of basal turn IHCs by several days (Kaltenbach and Falzarano 1994). Roth and Bruns (1992b) found a similar maturational gradient in the rat organ of Corti. Although a detailed study of the maturational gradient along the organ of Corti was not undertaken in this study, it was found that the apical surfaces of IHCs from the apical turn were less developed than those from the basal turn.

**B. The junctional region.**

The main finding of this section of the thesis is that the junctional region around the neck of the IHC is more mature than that of the OHC at 2 DAB, after which it matures more rapidly to attain a more complex mature junctional configuration than that found on OHCs. At a gross level, the junctional region at the apical pole of the IHC shares similar characteristics of tight and adherens junctions with OHCs as described and discussed above.

In contrast to the delayed maturation of the lateral membrane, the apical junctional region of the IHC appears to mature more rapidly than that of the OHC. Although the OHC
junction is subject to different factors and stresses, in particular the opening of spaces around the cell body and changes in the adjacent supporting cell morphology, the apicalmost junctional region of IHCs is more mature than the same region in the OHC as early as 2 DAB. By 8 DAB the apicalmost region had attained an almost mature form. However, in contrast to the OHC apicalmost region, the strands did not course in a circumferential ring around the neck of the cell. Rather, the lower strands extended into the basalmost region forming whorl-like patterns of junctional strands. The basalmost region continued to increase in complexity beyond this stage, similar to the OHC.

In the mature gerbil, the junctional region is more complex in the IHC than the OHC. Although not subject to the same stresses as the OHC, since the cell body is not totally surrounded by large spaces, there is a similar requirement for junctional sealing to maintain the integrity of the reticular lamina and the EP. The finding in this study that the tight junctional protein ZO-1 is localised at the IHC junctions at 2 DAB and does not appear to increase substantially in extent during maturation may suggest that in addition to performing a sealing function, the IHC apical junction may have a role in mechanically stabilising the apical membrane and the stereocilia. For example, the presence of a more advanced junctional configuration seen at 2 DAB may be related to the presence of larger and more robust stereocilia on the apices of the IHC. Why the junctions of mature OHCs and IHCs should be of differing configuration is unclear. The differing morphology of the junctions may be related to functional requirements which may be reflected in differences in the cell adhesion molecules which constitute the junctions.

In the assembly of tight junctions, E-cadherin is known to have a role in the initial cell-
cell contact stage (Gumbiner et al 1988). Cadherins are transmembrane glycoproteins normally associated with adherens junctions, and their role in junction formation and cell adhesion is discussed in detail below. Although found at the OHC lateral membrane during development in the mouse, E-cadherin is not found on the IHC lateral wall or junctional regions during development (Whitlon 1993). Whilst the presence of E-cadherin on OHC lateral walls may suggest a role in the process of opening the extracellular spaces around the OHCs as discussed below, the lack of spaces around the mature IHC would preclude the requirement of E-cadherin in this role. However, the lack of E-cadherin at any developmental stage as reported by Whitlon (1993) is surprising given its role in the assembly of tight junctions and the presence of the tight junctional protein ZO-1 at the IHC apical junctions at 2 DAB, as described in this study. Possibly another member of the cadherin superfamily of adhesion molecules occurs at the IHC junctional region, the expression of which may be involved in not only the junctional assembly but also changes in the lateral wall IMP distribution, since 8 DAB marks not only changes in the tight junctional region conformation but the appearance of plaque structures and increases in the number of IMPs organised in rows.

C. The lateral membrane.

Counts of the IMP density of the IHC lateral membrane show a complex sequence of changes. Initially, at 2 DAB, the lateral membrane is covered in large numbers of predominantly small protein particles. These subsequently increase in number, reaching a maximum about 10 DAB. From 10 DAB onward the total IMP count decreases, in particular the number of small particles. A different sequence of maturation appears to occur for the larger particles. At 2 DAB they appear randomly distributed about the membrane, and commence organising into straight rows of particles about 6 DAB. From
6 DAB onward, the large particles increase in number, forming many rows of particles some of which run parallel to each other. There does not, however, appear to be any overall pattern formed by the particle rows. A third feature of the IHC lateral membrane, patches or plaques of large IMPs organised in square arrays, do not appear until about 8 DAB. The plaques first appeared as small groups of particles, few in number and distributed over the cell membrane. Although the plaques increased in size and number subsequent to this, they had still not attained adult-like numbers by 16 DAB. These results indicate that substantial reorganisation of the IHC lateral membrane occurs in the period coinciding with the onset and maturation of auditory function. Immediately prior to the first stimulus evoked responses from the IHCs, as measured by the CAP, the number of rows of particles increased and the first appearance of plaques was noted. The loss of large numbers small particles from the membrane may be significant in suggesting that the number or type of membrane ion channels undergoes a significant change at this time.

The functional significance of both the rows of IMPs and the plaques is unclear. Forge (1987) suggested that the plaques were the site of transmembrane channels, since they were not junctional in character nor the sites of anchoring for cytoskeletal elements. Furthermore, the units composing the plaques span the membrane, in a similar manner to gap junctional units (Forge 1987). It is perhaps significant that the plaques appear to be last feature of the IHC lateral membrane to mature, some time after the onset of evoked responses.

One hypothesis would be that the plaques represent regions of ion channels devoted to
a rapid turnover of ions. That is, the ions entering the cell due to stimulation of the stereocilia are rapidly cycled out of the cell to restore the membrane potential and/or to prevent the IHC becoming too loaded with positive ions and thereby reducing sensitivity. The positioning of the plaques mainly toward to apex of the cell, close to the site of ion entry, may support this interpretation. Furthermore, the appearance and increase in numbers of the plaques corresponds to the onset of EP, when the level of $K^+$ passing into the IHC due to the increasing driving force is rapidly rising. The requirement to remove surplus ions from the interior of the IHC would therefore increase in parallel with the growth of EP. This proposed increasing efficiency of the IHC in maintaining its sensitivity by controlling the ionic concentration within the cell is further paralleled by the increasing sensitivity and response magnitude of the CAP from 12 DAB onward. The finding of Kros et al (1991), of increasing $K^+$ current outflow in IHCs in vitro coincident with the onset of hearing may be related to the changes in the IMP density of the IHC lateral membrane described in this study which occur over the same time period.

IHC have low resting potentials of approximately -40 mv (Russell and Sellick 1978) compared to supporting cells which have resting potentials of between -70 and -100 mv (Nuttall and Lawrence 1979). The low resting potential may be the result of a continuous influx of $K^+$ through the cell, the excess positive charge tending to depolarise the cell and cause the release of neurotransmitter at the afferent synapse. This would create a continuous background activity of spontaneous discharges in the afferent nerve (Kiang et al 1970, Liberman and Kiang 1978). That this spontaneous discharge is to some extent dependent upon the flow of $K^+$ into the IHCs has been shown by Sewell (1984), who showed that administration of furosemide, which reduces EP, alters the spontaneous
discharge rate. Other manipulations of EP, for example anoxia, have been shown to produce reductions in the IHC receptor potential which correlate with the time course of the decrease in EP (Brown et al 1983, Russell and Cowley 1983, Nuttall 1984). One may suggest that in order to control the non-stimulus related flow of ions into the IHC, and hence maintain the spontaneous discharge rate at an acceptable level, the IHC may have some mechanism of shunting excess K⁺ out of the cell. The presence of populations of IMP on the lateral wall may be indicative of sites of ion channels responsible for this maintenance system.

Although afferent synapses are already present on IHC basal membranes at 2 DAB, it is unlikely they are transmitting stimulus-derived information to the higher auditory centres. Many studies have shown that disruption of the afferent innervation during development leads to structural and functional deficits at higher levels of the auditory system (for example Silverman and Clopton 1977, Coleman et al 1982, Evans et al 1983). It is known that deprivation of sensory input leads to afferent fibre degeneration. Therefore, prior to the onset of EP, a small current through the IHC may be required in order to maintain the afferent nerves, via a constant spontaneous discharge. Since there is no EP driving current through the cell, K⁺, which is at mature concentrations in endolymph before the rise of EP (Bosher and Warren 1971), K⁺ ions may have to be allowed entry into the IHC via other routes in addition to the apical surface. The presence of large numbers of small IMP on the lateral membrane from 2-10 DAB may be indicative of a population of ion channels responsible for maintaining a positive ion entry into the cell causing spontaneous activity at the afferent synapse.
D. Summary II.

The maturation of the IHC membrane specialisations highlights a paradox in IHC development. Whilst the apical surface features of microvilli would suggest that IHC mature after OHC, the stereocilia and the junctional region around the neck of the cell is more mature than the OHC. In addition, changes in the IMP distribution on the lateral membrane also indicate a delayed maturation with respect to OHCs. Synaptogenesis, however, which was not studied in this thesis, is known to be complete at the IHC some time before the OHCs (Kikuchi and Hilding 1965, Lenoir et al 1980). This may be due to the change from afferent to mainly efferent innervation to the OHC, discussed above, whilst the IHC retains a primarily afferent innervation. This suggests that the changes in lateral membrane IMP configuration are to some extent independent of the acquisition of innervation.

Previous studies at the SEM and TEM level have suggested that IHC development ends with the onset of hearing (Roth and Bruns 1992b). However, the data presented in this study indicates that substantial changes to the IHC lateral wall continue to take place beyond the onset of CM, CAP and SFOAEs. The findings of this study suggest the following sequence of IHC maturation. The presence of larger and more mature stereocilia on the apices of the cell requires a more complex junction in order to stabilise the apical membrane. The lateral membrane is initially covered with large numbers of IMPs, randomly distributed. As the EP and K⁺ flow through the IHC increases, reorganisation of the lateral membrane occurs, possibly with a change in the type and distribution of ion channels. This change occurs during the period of rise in EP and the onset of cochlear function. The final maturation of the IHC lateral membrane coincides
with increasing sensitivity of cochlear responses to stimuli, suggesting that one factor responsible for the attainment of adult cochlear responses is the maturation of the IHC lateral membrane.

This study has shown that the lateral membrane of the gerbil IHC continues to mature throughout the period of onset of auditory function and during the time of increasing responses to auditory stimuli. It may be suggested therefore, that the reorganisation and relatively late maturation of the IHC lateral membrane, in particular the reorganisation of IMP distribution, is a major constraint on the development of mature cochlear evoked responses.

4.5 Junction formation and junctional protein expression.

The maintenance of the fluid barrier separating endolymph from perilymph relies on the integrity of the intercellular junctions between the constituent cells of the reticular lamina. Homotypic junctions exist between adjacent supporting cells and heterotypic junctions occur between hair cells and their adjacent supporting cells. These junctions are morphologically distinct, as noted previously. When examined using freeze fracture techniques the homotypic junctions between supporting cells consist of junctional strands which run parallel to the apical surface of the cells, a configuration which is indicative of tight junctions (Claude and Goodenough 1973, Iurato et al 1976). The heterotypic junctions between OHCs and supporting cells are more highly developed, consisting both of strands oriented parallel to the cell apical surface and below this a region of irregularly arranged strands which form polygonal arrays. The presence of two distinct morphologies of junctional strand arrangement have led to the suggestion that these junctions consist
of a very tight junction at the apical pole which lies above a region which may have an adherens function. In addition to the necessity of fluid sealing by the tight junction, mechanical coupling between the OHCs and supporting cells must withstand deformation due to the stimulus induced motion of the basilar membrane, as well as the motility produced by the OHCs.

The molecular candidates responsible for mediating intercellular contacts in epithelial tissue include the cadherin family, transmembrane glycoproteins which mediate Ca\(^{2+}\) dependent cell to cell adhesion in virtually all solid tissue (Takeichi 1991). Cadherin function at junctions involves the specific binding of homotypic extracellular domains and interactions with components of the cytoplasm (Gumbiner and McCrea 1993). In particular, they interact with actin filaments (Hirano et al 1987) via their anchoring proteins, the catenins (Ozawa et al 1990) which have been localized at adherens junctions (Geiger and Ayalon 1992). Long term effects of cadherin mediated cell adhesion include extensive reorganisation of the membrane and the cytoskeletal protein composition (Rodriguez-Boulan and Nelson 1989, Nathke et al 1993).

A member of the cadherin family, E-cadherin, facilitates the formation of both tight and adherens junctions (Gumbiner et al 1988) as well as playing a role in the organization of a polarized cytoskeleton and organelles (Gumbiner and McCrea 1993). Formation of both tight and adherens junctions is dependent upon the function of E-cadherin at the cell surface and inhibition of E-cadherin at the cell surface slows the formation of both junctional types (Gumbiner et al 1988). One model for E-cadherin function involves it in the first stage of cell recognition and adhesion, holding the cells close together to allow
the formation of other junctions. Once this has occurred, E-cadherin has a less importantole in cell to cell adhesion (Gumbiner et al 1988). Along with other members of the
cadherin family, for example N and P-cadherins, E-cadherin is implicated in processes
other than junction adhesion, for example cell signalling events. There is evidence that
G-protein pathways are activated by E-cadherin adhesion (Cereijido et al 1993) and
Ca^{2+} dependent adhesion also plays a role in the morphological regulation activities of
N-cadherin via G-protein activated Ca^{2+} channels (Doherty et al 1991). Further
evidence of second messenger mediated consequences of cadherin cell adhesion comes
from the involvement of protein kinase C, whose activation following cell adhesion
triggers the formation of tight junctional strands (Balda et al 1993) and has a regulatory
role in the assembly of adherens junctions (Lewis et al 1995). Other intracellular
signalling pathways, for example calmodulin mediated effects, may also be activated
following E-cadherin cell adhesion (Balda et al 1991).

In epithelial tissues, the most apical structure of the junctional complex is the tight
junction. The tight junction, as well as having a sealing function, also serves to separate
the plasma membrane into apical and basolateral domains, the protein and phospholipid
constituents being unique to each domain. Proteins associated with tight junctions are ZO-
1 (Anderson et al 1988), ZO-2 (Gumbiner et al 1991) and cingulin (Citi et al 1988). Ca^{2+}
dependent cell to cell contact, mediated through E-cadherin prior to the recruitment of
ZO-1 to the plasma membrane (Siliciano and Goodenough 1988), triggers activation of
a protein kinase C pathway which in turn regulates the assembly and sealing of tight
junctions (Balda et al 1991, 1993). Thus, E-cadherin occurs in regions which become
tight junctions prior to the appearance of the tight junctional proteins.
Adherens junctions, as noted above, contain cadherins as their major "contact receptors" (Geiger and Ayalon 1992). Cell to cell adhesion usually occurs between the extracellular domains of E-cadherin molecules present on the surface of both cells. However, switches in cadherin expression are known to occur during development, for example in neural tube development where the initial expression of E-cadherin gives way to N-cadherin expression. Thus, the expression of adhesion molecules appears to be under precise spacial and temporal control and is associated with a variety of morphogenetic events during development.

A. The inner ear.

Much of what is known of the distribution of cadherins in the inner ear comes from work on the chick basilar papilla. During the early stages of development the chick homologue of E-cadherin, LCAM, (Gallin et al 1983), has been found to be co-expressed in the epithelium which becomes the basilar papilla with ACAM, the homologue of N-cadherin (Volk and Geiger 1984). Following differentiation, ACAM becomes restricted to the sensory region of the basilar papilla with LCAM being expressed throughout the non sensory regions (Richardson et al 1987, Raphael et al 1988). ACAM is found in both homotypic and heterotypic junctions. This differs from the situation as at present thought to pertain in the mammalian organ of Corti. The study of Whitlon (1993) represents the only current data on E-cadherin distribution in the organ of Corti. This study showed that in the mature mouse organ of Corti, E-cadherin was expressed only at homotypic supporting cell to supporting cell junctions of the reticular lamina, and was not present at the heterotypic OHC to supporting cell junction. A different pattern of expression was, however found in the developing organ of Corti. During development, E-cadherin was found to be transiently expressed along the lateral wall of the OHCs and the neighbouring
supporting cells, as well as at the OHC cell apices. There was no expression at the IHC junctional regions. The lateral wall distribution was lost at the same time as the spaces around the OHCs began to open, the distribution at the cell apical region being lost slightly later (Whitlon 1993). These data would imply that the homotypic supporting cell to supporting junctions and the heterotypic OHC and IHC to supporting cell junctions have not only differing morphologies, as noted above, but also differ in the identity of their "molecular contact receptors" (Geiger and Ayalon 1992). Given the presence of the N-cadherin homologue in both homotypic and heterotypic junctions of the avian basilar papilla (Richardson et al 1987, Raphael et al 1988), N-cadherin would be a candidate for the adherens junctional molecule at the mature OHC junction and the IHC junction. However, a report by Nunes et al (1996) failed to find either N or P-cadherins in the mature organ of Corti junctions.

Catenins, as noted above, mediate the cadherin-actin filament interactions in adherens junctions. However, Nunes et al (1996) failed to localise α or β-catenin at hair cell junctions in the adult guinea pig organ of Corti. Both molecules were however localised at supporting cell-supporting cell junctions (Nunes et al 1996). Other known cytoplasmic proteins which are associated with cadherins and which have been identified to date in the OHC include cingulin, which is present in junctional regions of OHCs in mature guinea pig (Raphael and Altschuler 1991, Citi 1993). Cingulin possibly interacts with actin filaments which are present in the cuticular plate. Since maturation of the cuticular plate is coincident with the development of the basal junctional region in the gerbil OHC, an increase in cingulin and other adherens junction associated proteins may occur at this time. Given that E-cadherin is not expressed in the mature OHC in mice (Whitlon 1993),
and that this study shows that junctional development continues after E-cadherin expression has been reported as ending (Whitlon 1993), it may be suggested that the junctional element will be found to be another of the cadherin family, as yet unidentified in the gerbil OHC.

Lewis et al (1995) have hypothesised that E-cadherin has a role in mediating Ca2+ dependent homotypic adhesion between cells and also, via an intracellular signal that activates protein kinase C, leads to the organisation of junctional components. Whilst Whitlon (1993) suggested that down-regulation of E-cadherin facilitated the opening of the fluid spaces, it is also possible that E-cadherin may have a role in initiating the assembly of the junctional components prior to this, as well as promoting, as a consequence of down-regulation, the changes in the OHC lateral wall which occur around the same time as space opening, for example the laying down of the cisternae and/ or the assembly of the OHC cortical cytoskeleton.

In this thesis, data is presented which shows the tight junctional protein ZO-1 to present at all cell to cell junctions of the reticular lamina, including hair cell- supporting cell junctions, from 2 DAB onward. Thin sections and freeze fracture replicas also show that both tight and adherens junction regions continue to enlarge up to about 8 DAB for tight junctions and up to 16 DAB for the adherens region. As noted above, the reported lack of E-cadherin at the maturing IHC junctions is surprising given the role of E-cadherin in the assembly of tight junctions, since blocking the adhesive function of E-cadherin prevents the assembly of tight junctions (Gumbiner et al 1988). Furthermore, cells with low levels of E-cadherin expression fail to distribute the ZO-1 tight junctional protein to
the membrane (Rajasekaran et al 1996). The lack of both E-cadherins and \( \alpha \) or \( \beta \)-catenins at the OHC and IHC junctions may suggest a different pathway is involved in the assembly of the hair cell-supporting cell tight and adherens junctions in the organ of Corti than is commonly found in epithelial tissue, which relies on E-cadherin and catenin-ZO-1 complexes (Gumbiner and M\'Crea 1993). This interpretation is supported by the morphologically distinct nature of the junctional complexes of the hair cells as revealed by freeze fracture replicas of the hair cell membranes.

The identity and sequence of expression of the adhesion molecule at the OHC to supporting cell junction is of importance with regard to repair of the sensory epithelium following damage. Studies have shown that following damage to the organ of Corti, the most severely affected OHCs degenerate, to be replaced by a scar, formed as the adjacent supporting cells expand to fill the gap and maintain the sealing of the reticular lamina (Forge 1985, McDowell et al 1989, Raphael and Altschuler 1988). This scar formation necessitates a supporting cell forming a new junction with another supporting cell, consisting of a tight and adherens junction (Raphael and Altschuler 1991). There is also evidence that a similar process may be involved in damage repair in the vestibular system (Li et al 1995).

The characteristics of the adhesion molecules at the reticular lamina may also have implications for putative regenerative processes in the mature mammalian organ of Corti. Whilst the reappearance of sensory cells following damage has been observed in mammalian vestibular tissue in vivo and in vitro (Forge et al 1993a, Warchol et al 1993), and regeneration of sensory cells has also been described in the avian basilar papilla.
following damage (Cotanche 1987), there is no evidence as yet of similar replacement of sensory cells occurring in the adult mammalian organ of Corti. Sensory cells of both the vestibular organ and the avian basilar papilla are surrounded by supporting cells, with no spaces between the cell bodies. As noted above, in the avian basilar papilla, both homotypic and heterotypic junctions express the homologue of N-cadherin. There is currently no information available on the identity of the cell adhesion molecule in the vestibular epithelium. If, as suggested above, the OHC to supporting cell junction is composed of different adhesion molecules during the course of development and in the mature state, and this changing expression is also associated with other OHC morphological changes during the developmental sequence, then one difficulty facing potential organ of Corti sensory cell regeneration may reside in the precise spacio-temporal regulation of expression of the adhesion molecules and the possible necessity of having to "recapitulate" the correct sequence of adhesion molecule expression, and morphological rearrangement to produce OHCs in the correct anatomical and spacial environment.

4.6 The stria vascularis.

The gross morphological maturation of the stria vascularis of the gerbil described in this thesis proceeds in a similar manner as that described in the mouse (Kikuchi and Hilding 1966); rat (Rybak et al 1991); rabbit (Anggard 1965); cat (Fernandez and Hinojosa 1974) and humans (Lavigne-Rebillard and Bagger-Sjöback 1992). A sequential appearance of marginal cells, intermediate cells and finally basal cells appears common in the mammalian species studied. The results obtained for the onset and maturation of the EP are also in accord with previous studies in the gerbil (Ryan and Harris 1986, McGuirt 324
et al 1995) and other rodents (For example Rybak et al 1992).

The studies presented in this thesis have concentrated upon the maturation of junctional specialisations of the marginal and basal cells. Together with the study of Na\(^+\)-K\(^+\) ATPase reactivity and the onset and maturation of the EP, the aim of these studies was to identify possible morphological features whose appearance and maturation coincided with the onset and maturation of the EP recorded from the scala media.

A. Na\(^+\)-K\(^+\) ATPase activity.

A small amount of staining for Na\(^+\)-K\(^+\) ATPase reactivity was found on the basolateral walls of the marginal cells at 2 DAB. By 6 DAB this had increased substantially and was found at adult-like levels by 12 DAB. Although the appearance of substantial enzymatic activity coincides with the onset of the EP measured in this study, EP continued to rise well beyond the time when the Na\(^+\)-K\(^+\) ATPase activity appeared to be at adult levels. This suggests that the Na\(^+\)-K\(^+\) ATPase present on the basolateral walls is not the only contributor to EP generation. Na\(^+\)-K\(^+\) ATPase activity has previously been studied in the developing rat cochlea. Kuijpers (1974) found enzymatic activity to be present from 6 DAB onward, reaching mature levels after 18 DAB, coinciding with the attainment of functional maturity. This is supported by studies of Yao et al (1994), who found that the Na\(^+\)-K\(^+\) ATPase \(\alpha\)1 subunit mRNA was present from 1 DAB in the rat stria, reaching adult levels by 12 DAB, coincident with the rise of EP. Further evidence that the Na\(^+\)-K\(^+\) ATPase enzyme is not the sole determinant of EP generation comes from studies of the Viable spotted dominant mutant mouse, which although having apparently morphologically normal marginal cells (Steel et al 1987) and normal levels of Na\(^+\)-K\(^+\) ATPase activity (Schulte and Steel 1994), does not posses an EP.
Freeze fracture replicas of the lateral membrane show increasing IMP density between 2 and 6 DAB. This provides circumstantial evidence that the IMP are related to sites of the Na\(^+-\)K\(^+\) ATPase enzyme, since the increase of the enzyme reactivity seen in thin sections occurs over the same time period.

**B. Marginal cells.**

At 2 DAB the junctional region around the apical pole of the marginal cells was already well developed. The apicalmost region consisted of up to 4 parallel strands, indicating a high degree of sealing was already present at this stage. From 6 DAB onward, the strands from the tight junctional region coursed into the more basal region, in a similar manner to that observed in IHCs in the organ of Corti. These strands did not appear to make contacts with the more eccentrically coursing junctional strands of the basalmost junctional region. The apicalmost region exhibited an adult-like appearance by 12 DAB, however the total junctional depth continued to increase beyond 16 DAB. This suggests that the sealing function of the junction at the luminal pole of the marginal cell is fully established by the onset of the EP. Furthermore, since the tight junction appears well established before the period of EP rise, the maturation of the junctions at the luminal pole of the marginal cell are unlikely to be a factor in the onset and rise of the EP.

It appears that the ionic composition of endolymph, in particular the K\(^+\) levels, and the generation and maintenance of EP are to some extent separate. Bosher and Warren (1971) found that the endolymphatic ion concentrations were established at mature levels before the onset of EP. This suggested that the two arise to a large degree independently of each other. The studies reviewed in the Chapter 1 which showed that reductions of K\(^+\) levels produced by ouabain and furosemide differed although the levels of EP were decreased
by the same amount (for example Sterkers et al 1988, Shugyo et al 1990) support this interpretation.

The maturation of the marginal cells, in particular the acquisition of adult-like levels of Na⁺-K⁺ ATPase at the basolateral wall prior to the onset of EP, may be necessary to establish the ionic content of endolymph. The results of this study showing that the apical junctional region and the levels of Na⁺-K⁺ ATPase reactivity are mature prior to the period of increasing EP suggests such a role for the marginal cells in maintaining endolymph composition. However, if the generation and maintenance of the EP is also dependent upon the maturation of the tight and gap junctions of the basal cells, the onset and rapid rise of EP would be expected to be more closely correlated with basal cell junctional maturation.

C. Basal cell tight junctions.

Identifiable basal cells did not appear before 4 DAB. At 4 and 6 DAB a few broken tight junctional strands were present, in no apparent relationship to each other. By 8 DAB, the number of strands per \( \mu m^2 \) had more than doubled and by 10 DAB the junctional strands extended over large areas of the cell membrane. The strands had also organised into numerous rows of up to 6 parallel strands, indicative of a strong sealing capability, and regions of gap junction-like particles had appeared between some of the tight junctional strands. Therefore, just prior and coinciding to the onset of EP, rapid changes in the basal cell membrane had occurred which would effectively have begun to seal the stria from the perilymph of the spiral ligament. This tight junctional region continued to increase in complexity beyond 18 DAB, as shown by the presence of broken, immature junctional strands at 18 DAB. This suggests that sealing of the strial compartment
continued to increase during the period of EP maturation.

**D. Basal cell gap junctions.**

Patches of small gap junctions did not appear on basal cells until 6 DAB, and by 8 DAB they still occupied a small area of the basal cell membrane. By 12 DAB, the onset of EP, the area occupied by the gap junctions had increased dramatically. Between 12 and 16 DAB, the gap junctions continued to increase in size and number, and by 18 DAB had rearranged into large, well defined gap junctional patches.

The appearance of large areas of gap junctions coincident with the onset and period of rapid rise in EP, together with the increasing complexity of the basal cell tight junctions over the same period, suggests that the formation of the basal cell junctional specialisations may be a major contributor to the establishment of the EP.

**E. Basal cell junctional maturation and EP onset and maturation.**

Kikuchi et al (1995) described two gap junction systems within the cochlea; an epithelial gap junction system and a connective tissue system. The former consists of the supporting cells of the organ of Corti and root cells which extend into the fibrocytes of the spiral ligament which underlie the stria and form part of the connective tissue gap junctional system. The two systems are in contact via gap junctions.

It is thought that $K^+$ is released into the extracellular space of the organ of Corti following stimulation of the hair cells. This requires some mechanism to buffer or remove the excess $K^+$ in order to maintain pH and allow the hair cells to return to their resting potential. Santos-Sacchi (1991) suggested that supporting cells may act to buffer $K^+$ levels by taking up the extracellular $K^+$ released during stimulation. According to the
model of Kikuchi et al (1995), the K⁺ ions would then be conveyed down a concentration
gradient, away from the hair cell region, to the root cells which form one end of the
epithelial gap junctional system. K⁺ ions expelled from the root cells are taken up by the
fibrocytes of the connective tissue gap junctional system which in turn are connected to
the strial basal cells by gap junctions. The K⁺ would then be passed through the stria to
emerge in endolymph via the intermediate and marginal cells (Kikuchi et al 1995). The
presence of the tight junctions on basal cells would serve to isolate the stria from the
perilymph of the spiral ligament, allowing only cell-cell transport of ions via gap
junctions. The tight junctions would also serve to electrically isolate the stria allowing a
potential difference to build up.

The findings of this study, which suggest that the generation of EP is dependent upon the
maturation of tight and gap junctions of the basal cells, would be in accord with a model
of K⁺ cycling within the cochlea as described above. The basal cell tight junctions would
need to provide effective sealing of the body of the stria from the perilymph in order to
establish a K⁺ gradient, whilst a functional basal cell gap junctional system would be
required to allow the K⁺ entry from the fibrocyte layer below. This study shows that the
appearance of tight junctions coincides with the onset of EP, whilst the basal cell
membrane is covered with large numbers of developing gap junctions at this stage and
during the period of EP rise. Although circumstantial, the data showing the maturation
of both gap and tight junctions on basal cell membranes to coincide with the onset and
rise of EP suggests that the maturation of these membrane specialisations is perhaps the
final, major event in the maturation of the stria and fundamental to the onset and
generation of the EP.
The findings of this study reveal a complex sequential pattern to the maturation of the structures of the organ of Corti, the stria vascularis and the stimulus evoked potentials. Although it is not possible to state definitively that one event is dependent upon another, there is circumstantial evidence to indicate that the acquisition of certain characteristics and specialisations of cells of the organ of Corti and the stria vascularis are crucial to the attainment of a normal functioning cochlea.

Principally, the finding that on both a gross morphological level and at the level of cell membrane specialisations that mature characteristics are not attained until well after the onset of hearing, as measured by CAP and SFOAE responses, provides evidence that the increasing sensitivity at the auditory nerve level has its origin in the maturation of peripheral structural features. The main findings of this study are summarised below.

1. The results of the morphological study of the organ of Corti established the presence of two maturational gradients. A sensory cell gradient, with IHC and OHC maturation proceeding from base to apex and an accessory structure gradient starting in the middle turn before extending to the basal and finally apical turn of the cochlea. The major morphological event, the opening of the extracellular spaces of the organ of Corti was first observed in the middle turn, occurring between 8 and 10 DAB. This was found to coincide with changes in the lateral wall of OHCs.
2. At the gross morphological level, the maturation of accessory structures, in particular the supporting cells and the basilar membrane, continued after the onset of hearing. This may have implications for the cochlea's response to high and low frequencies which is reflected in changes in the cochlear place code map which is known to undergo changes in the developing gerbil.

3. In this study it has been shown that the lateral membrane specialisations of the OHCs are acquired sequentially. IMPs, which may represent the sites of ion channels or the putative motor elements of the OHC, progressively increased in number from 2 DAB onward, reaching adult-like numbers by 16 DAB. The sub-surface cisternal network, together with its associated pillar structures, did not appear until after motile responses can be elicited from isolated gerbil OHCs (He et al 1994). This finding supports the hypothesis that the OHC motor elements are located at the basolateral membrane of the OHC and not at the cisternal network.

4. This study has shown that the maturation of the IHC continued beyond the onset of hearing and during the period of increasing cochlear response sensitivity. The lateral membrane of the IHC was found to undergo a complex sequence of changes during maturation. Initially covered with large numbers of randomly organised large and small particles, the total number of IMPs declined after 10 DAB. From about 6 DAB onward the large particles organised into rows, whilst plaque structures did not appear until 8 DAB and remained at an immature number up to 18 DAB. The decline in the total IMP count was apparently due to loss of the small particles. It was suggested that this IMP reorganisation was related to the establishment of EP and consequent changes in the ionic flow through the IHCs. The delayed maturation of the IHC lateral wall will also have consequences for the activation of afferent nerve synapses and hence the auditory nerve
response maturation.

5. Although the apical surface of OHC appeared to mature before the IHCs, as defined by loss of microvilli, at 2 DAB the IHC stereocilia appeared more mature than those of OHCs. Furthermore, in contrast to the later maturation of the IHC lateral membrane, the junctional region around the neck of the IHC was more complex and matured before that of the OHC. The greater complexity of the IHC junction may be related to increased loading created by the larger stereocilia, whilst the delayed OHC junctional maturation may be related to changes in supporting cell morphology due to the opening of the extracellular spaces.

6. The results of the study of the maturation of the stria vascularis have provided evidence that the major limiting factor in the onset and maintenance of the EP is the appearance and maturation of the tight and gap junctional complexes of the basal cells. Levels of Na⁺-K⁺ ATPase activity at the marginal cell lateral wall were found to be at adult-like levels by 12 DAB. Therefore, since maturation of the marginal cells was found to be substantially complete by 12 DAB, these cell are unlikely to be the determining factor in EP maturation. The appearance of the basal cell junctional specialisations was discussed in regard to a model of K⁺ ion flow through the cochlea (Kikuchi et al 1995), the maturation of the basal cell junctions being a crucial stage in such a model. It was noted that the onset of EP also coincided with the appearance of plaque structures on the lateral membrane of IHCs, the decrease in numbers of small IMPs from this membrane and the reorganisation of larger IMPs into rows.

7. The onset and maturation of stimulus evoked potentials and SFOAEs was found to accord with previous studies. In this study it was possible to relate the onset and growth of these responses with changes at the morphological level. It was suggested that the
delayed maturation of the basilar membrane could account for a proportion of the reduced response magnitude. Other factors, such as the maturation of the OHC and IHC lateral membranes, the maturation of strial basal cell junctions and their influence on EP generation, would also have implications for the maturation of hearing.

Although temporally related, the maturation of the various elements of the cochlea summarised above may in fact be independent of each other. It may be suggested that the maturation of junctional and lateral membrane specialisations of OHCs are related to mechanisms involved in the opening of the organ of Corti spaces. Separation of the OHC from close apposition with the lateral wall of the pillar cells is coincident with major structural changes in the OHC lateral wall. The trigger for these changes may be changes in the expression of cell adhesion molecules. In the case of the IHC membrane specialisations, different factors may be involved. The more complex and early maturation of the IHC apical junction may be related to the greater loading of the IHC stereocilia. The complex sequence of lateral membrane IMP changes are possibly related to the onset of EP and the establishment of a greater current flow through the cell. The maturation of the marginal cells of the stria, and adult-like levels of Na⁺-K⁺ ATPase activity, occurs before the rapid rise in EP, suggesting that the marginal cells are involved in maintaining the K⁺ concentration of the endolymph. The maturation of the junctional specialisations of the basal cells occurs coincident with the establishment and rise of EP, suggesting that the basal cell tight and gap junctions are important factors in EP generation. One might speculate whether the IHC lateral membrane specialisations would reach a mature state in animals which lacked an EP due to strial basal cell loss.
The maturation of the sensory cells has obvious implications for the cochlea's ability to analyse incoming acoustic stimuli effectively. However, normal functioning also requires analysis of the acoustic stimulus by the basilar membrane, and normal coupling of the output of the OHC motor elements back into the basilar membrane vibration. These functions would be impaired by the delayed maturation of the basilar membrane and supporting cells described in this study.

CONCLUSION.

This study has identified four major elements in the maturation of the gerbil cochlea: the gross structural maturation of the basilar membrane and supporting cells which determine the coupling of the stimulus vibrations to the sensory cells of the organ of Corti; the maturation of the OHCs lateral membrane specialisations, including the putative OHC motor elements, which may be triggered by factors involved in the opening of the extracellular spaces; the onset and rise of EP, which appears dependent upon the maturation of basal cell gap and tight junctional complexes; and the maturation of the IHC lateral membrane which will influence the signal at the auditory nerve synapse, and hence the information conveyed to higher brain centres, and which may be dependent upon the onset and rise in EP.

The interdependence of these events is speculative, although their close temporal relationship may imply some degree of interdependence. This question could be resolved using techniques which would interrupt in some way the relationships between these elements, for example disrupting the function of adhesion molecules at the pillar cell lateral membranes to delay or accelerate the opening of the extracellular spaces, although
it may prove difficult to separate out the indirect, unwanted consequences of such manipulations.
REFERENCES.


Rajan, R. and Johnstone, B.M. (1983) Crossed cochlear influences on monaural temporary threshold shifts. Hearing Res. 9, 279-


Yancey, C. and Dallos, P. (1985) Ontogenic changes in cochlear characteristic frequency at a basal turn location as reflected in the summating potential. Hearing Res. 18, 189-195.


