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THE RETINAL PATHOLOGY OF OVINE CEROID-LIPOFUSCINOSIS

A thesis presented in fulfilment  
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at Massey University

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## ABSTRACT

Ovine ceroid-lipofuscinosis is an animal model of a rare genetic disease of man and some domestic animals. The disease is characterized by blindness idiocy and premature death.

In order to investigate the development of pathological changes in this model it was important to be able to accurately diagnose those sheep affected at an early age before clinical disease was apparent. In this thesis a number of methods such as skin biopsy, bone marrow examination and brain biopsy were investigated and it was concluded that brain biopsy was the most suitable and reliable method for establishing the preclinical diagnosis of ceroid-lipofuscinosis.

On clinical grounds the ovine disease most closely resembled the juvenile form of human disease and blindness was the important clinical finding in both diseases. A time course study of the development of retinal pathology was carried out.

Electroretinography was used as a clinical tool to ascertain the functional status of the retina. Pathological changes to the retina were investigated using light and electron microscopy.

Electroretinography revealed a decline in rod and cone 'b' wave amplitudes over a relatively short time span. Changes to the rod responses preceded, and were generally more dramatic than those of the cones. These changes paralleled a loss of rod and cone photoreceptor cells. Although there was some variation between animals and between readings it was suggested that

electroretinography was a useful method of monitoring changes to the retina and may be useful in assessing therapeutic strategies.

In affected retinas photoreceptor cell outer segments appeared to be shorter than those of controls. By 84 weeks of age the outer nuclear layer was reduced to a single row of nuclei with only remnants of outer segments present. Electron microscopy confirmed these findings and showed that the formation of abnormal dystrophic outer segments of photoreceptor cells was a significant early pathological change. Most cell types in the retina contained autofluorescent lipopigment bodies in their cytoplasm with the ganglion cells usually containing the largest amount. Ultrastructural studies showed that the storage bodies were made up of electron dense granular material and a variety of membranous and tubular structures giving them a similar appearance to those which were reported in the central nervous system. Very small electron dense 'smudges' were seen in the cytoplasm of some cells and these may have been early storage body precursors.

This study showed that electroretinography could be used to monitor the development of blindness in ovine ceroid-lipofuscinosis. It also revealed early severe pathological changes to the photoreceptor outer segments which may be of pathogenic significance in ovine and juvenile human ceroid-lipofuscinosis and therefore worthy of further investigation.

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## INTRODUCTION

The ceroid-lipofuscinoses are a group of rare genetic diseases of man and some domestic animals. Ovine ceroid-lipofuscinosis most closely resembled the juvenile form of human disease and like that condition blindness was the common early presenting sign. Little was known regarding the development of retinal lesions in these diseases because, of necessity, most studies have investigated patients with long standing disease and therefore advanced pathology. In the well-developed canine model retinal pathology was not a feature.

The purpose of this study was to investigate the time course development of retinal pathology in ovine ceroid-lipofuscinosis. Clinical changes were monitored by electroretinography and the pathological alterations were examined by light and electron microscopy. In this type of study accurate preclinical diagnosis of the disease was necessary so a variety of diagnostic methods were also investigated.

## CHAPTER 1

## A REVIEW OF THE RETINAL PATHOLOGY OF THE CEROID-LIPOFUCINOSES

## Historical

The neuronal ceroid-lipofuscinoses are a group of inherited diseases of humans and animals characterised by the intracellular storage of fluorescent lipopigment in neurones and a wide variety of other cell types. Clinically there tended to be blindness, progressive psychomotor disturbances, idiocy and premature death.

The nomenclature associated with this group of diseases has been confused. The earliest report in the literature was that of Stengel (1826), but Batten (1903) is generally acknowledged as providing the first comprehensive description of the disease. In 1905 two German neuropathologists Vogt and Spielmeier, independently described a disease of children which they called 'the juvenile form of amaurotic familial idiocy' and 'juvenile amaurotic familial idiocy' respectively. These early reports led to the eponymic names of Batten's or Batten-Spielmeier-Vogt disease for the condition. The term 'amaurotic familial idiocy' had been previously introduced by Sachs (1896) to describe another type of disease which came to be known as Tay-Sachs disease. For a number of years Batten and Spielmeier believed that their cases were different from Tay-Sachs disease but they eventually followed the unitarian concept which placed the neuronal ceroid-lipofuscinoses, Tay-Sachs disease and some

other lipidoses in a group known as the 'amaurotic familial idiocies'.

Other distinct subgroups were added to the amaurotic familial idiocies. A late infantile form was described by Bielschowsky in 1913 who considered his cases to be similar to those described by Jansky in 1908. An adult form with much less severe symptoms was described by Kufs in 1925. By this time all the classical sub groups had been identified but they were still considered to be forms of amaurotic familial idiocy.

In 1931 Sjorgren reported a further 115 cases of what is now known as the juvenile type and decided that his cases were distinctly different from Tay-Sachs disease. However, his report had little effect and the unitarian concept which held that these diseases were merely age variants of one pathological process continued until some 15 years ago.

Modern techniques of biochemistry and electron microscopy have led to the elucidation of the pathogenesis of some members of this group. The biochemical lesion of Tay-Sachs disease has been identified as a deficiency of hexoseaminidase A, leading to the accumulation of GM2 gangliosides (Okada and O'Brien, 1969). This discovery helped identify Tay-Sachs disease as a separate entity. Zeman and Dyken (1969) noted that, in the amaurotic familial idiocies, the accumulated lipopigments shared tinctorial and ultrastructural properties with ceroid and lipofuscin and introduced the name neuronal ceroid-lipofuscinosis for the group. Despite the designation that these were neuronal ceroid-lipofuscinoses they, in fact, represent generalised metabolic disturbances with many cell

types involved (Anzil et al., 1975; Jolly et al., 1980; Joosten et al., 1973; Schwendemann, 1976).

Originally the term ceroid-lipofuscinosis embraced the juvenile (Spielmeyer-Sjorgen), late infantile (Jansky-Bielchowsky) and adult (Kufs) forms, but the group has since been increased to include some important atypical forms (Goebel et al., 1976; Lake and Cavanagh, 1978; Libert et al., 1982). The development of a precise and rational system of nomenclature for the entities awaits the elucidation of the biochemical defect(s) involved.

Diseases characterized by the intracytoplasmic accumulation of lipopigments which are similar to those found in human ceroid-lipofuscinosis have been reported from several domestic animal species. Breeds of dogs in which cases have occurred include English Setters (Koppang, 1970, 1973/74, 1982), Chihuahuas (Rac and Griesecke, 1975), Dachshunds (Cumming and de Lahunta, 1977; Vandavelde and Fatzer, 1980) and Saluki's (Appleby et al., 1982). The disease has also been recorded from Siamese cats (Green and Little, 1974), an inbred strain of Beefmaster cattle (Read and Bridges, 1969) and an inbred flock of South Hampshire sheep (Jolly and West, 1976; Jolly et al., 1980; 1982). Of the animal cases, only the English Setter dogs and the South Hampshire sheep have been investigated in depth and colonies of these animals are maintained to provide models for ceroid-lipofuscinosis research.

#### Clinical Features

The principal clinical findings of the ceroid-lipofuscinoses were a progressive loss of mental and motor functions, blindness and premature death. The age of onset of the various clinical features

has been used to subdivide the human ceroid-lipofuscinoses into the major sub-types.

Ophthalmological findings:

Loss of vision occurred in all but the adult form of human ceroid-lipofuscinosis but the stage of the disease process at which this occurred varied among the sub groups.

The ophthalmological findings in patients with the infantile form of ceroid-lipofuscinosis have been reported (Raitta and Santavuori, 1973; Santavuori et al., 1973; Santavuori et al., 1974; Haynes et al., 1979 and Santavuori, 1982. Visual abnormalities were present in all cases but the degree of visual loss depended on the age of the individual when examined. One child when first examined at 18 months of age had normal visual function and a normal fundus, but was completely blind 17 months later. Typical ophthalmoscopic findings were quoted as optic atrophy, attenuation of the retinal vasculature, a dark brown macula and hypopigmentation of the peripheral fundus leading to a clearly visible choroidal circulation. Pigment aggregations and accumulations did not occur at the macula or in the peripheral fundus.

In most cases where electroretinography was carried out a response could not be recorded. In two patients, one aged 22 months and the other 5 years and 10 months, an electroretinogram was recordable (Pampiglione and Harden, 1974; Haynes et al., 1979). The responses had a normal appearance but there was a marked reduction in the amplitudes and latencies of the 'a' and 'b' waves. The younger patient had a non-recordable ERG when tested 3 years after the initial recordings were made.

Zeman et al., (1970) listed optic atrophy, attenuation of the retinal vasculature and macula pigment changes in 11 of their 15 patients classified as having the late infantile form of the disease. These general findings have been confirmed in later reports (Dolman and Chang, 1972; Harden et al., 1973; Goebel et al., 1977; Schochet et al., 1980; Warburg, 1982). In some cases the macula was normal. In this form of ceroid-lipofuscinosis blindness developed late in the course of the disease, usually after the patient had severe mental and motor disabilities. A recordable ERG with normal appearance and latency but reduced amplitude was usually found in these patients. However, later in the disease process the ERG became extinguished. Despite reduction or extinction of the ERG there was a gross enlargement of the visual evoked responses (Harden et al., 1973; Goebel et al., 1977; Harden and Pampiglione, 1982; Tackmann and Kuhlendahl, 1979). This combination has not been seen in other forms of the disease and as such may be a useful diagnostic aid.

In contrast to the other forms of the disease blindness was the common presenting sign in juvenile ceroid-lipofuscinosis (Zeman and Siakotos, 1973; Hansen, 1973; Spalton et al., 1980; Warburg, 1982). Failing vision was the presenting symptom in all but one of 26 cases reported by Spalton et al., (1980). The common age of onset was 6-7 years and blindness was complete in a further 1-2 years. Motor disturbances usually occurred some 2-4 years after the development of visual failure and it was significant that the diagnosis of ceroid-lipofuscinosis was usually not made until this time. Typical ophthalmoscopic findings were described as "bullseye" macula dystrophy (a ring of hypopigmentation surrounding the macula), attenuation of the retinal vessels and atrophy of the optic disc

(Zeman and Siakotos, 1973; Spalton et al., 1980; Warburg, 1982). The degree of macula pigmentary change was quite variable and many of the early changes were subtle and easily overlooked. The appearance of the peripheral fundus was also variable and ranged from normal to hyperpigmented. These changes and the development of so called 'bone spicules' occurred late in the disease process.

Hansen (1979) reported on the electrophysiological findings in seven cases of juvenile disease and in only one case was an ERG recordable. In this patient the amplitude of the 'b' wave was much reduced and a low amplitude 'flicker' ERG was recorded. It was postulated that the blue sensitive cones were still functioning and colour vision testing supported this hypothesis. Fluorescein angiography usually showed leakage of dye from the retinal vessels indicating active retinal degeneration (Spalton et al., 1980).

There are no reports in the literature of fundal abnormalities in the adult form of ceroid-lipofuscinosis even though retinal pathology was reported from one atypical case (Dom et al., 1979).

#### Mental and motor dysfunctions

Zeman et al., (1970) regarded mental and motor dysfunction as one of the obligatory signs in all forms of ceroid-lipofuscinosis. The age of onset varied but progression to more severe deterioration was inevitable. The infantile form was characterized by rapid loss of mental and motor function leading to ataxia, hypotonia and frequent myoclonal jerks. Once this stage was reached the patient sometimes lingered on for several years (Haltia et al., 1973 a, b ; Santavuori et al., 1973; Santavuori, 1982).

The infantile form, with an equally pernicious course, was usually complicated by the development of massive seizures which often resulted in death after a relatively short illness (Zeman and Dyken, 1969; Warburg, 1982). The juvenile form was usually first associated with the development of visual abnormalities but in some cases this was preceded by minor psychomotor disturbances. Major motor disturbances and seizures occurred later in the disease (Zeman et al., 1970; Zeman, 1976; Warburg, 1982). This entity often had a protracted clinical course with an average duration of illness of 11.5 years.

Mental abnormalities were much less severe in the adult form and did not affect all mental faculties. Dementia was never severe and motor abnormalities were confined to cerebellar ataxia, stiffness and akinesia (Zeman et al., 1970).

Electroencephalographic abnormalities occurred in the various subgroups and serial recordings reflected the continual deterioration of mental functions. Neurophysiological studies on 88 children with various forms of ceroid-lipofuscinosis showed that in general, the infantile form was characterized by a progressive decrease in amplitude of the electroencephalogram (EEG) while polyphasic spike discharges and large amplitude irregular slow activity occurred at low rates of photic stimulation in patients with the late infantile form. Juvenile disease was usually marked by runs of slow spike and wave activity in the EEG (Harden and Pampiglione, 1982; Pampiglione and Harden, 1977).

### Pathological Findings

The name of this group of diseases implies that the neurones were primarily involved but most tissues and cells in the body have been found to harbour typical lipopigments. However it was only in the neurones, including those of the retina, that the formation and accumulation of the storage product was associated with significant degenerative changes and the development of the characteristic clinical signs.

#### Clinical pathology

A number of abnormalities of leucocytes have been reported from cases of human ceroid-lipofuscinosis. Lymphocyte vacuolation, first reported in the juvenile form by Bagh and Hortling (1948), always occurred in this group (Dolman et al., 1975; Nakano et al., 1979; Noonan et al., 1978). It does not occur in the infantile disease (Santavuori et al., 1973; Warburg, 1982) and was a variable finding in late infantile cases (Lake, 1977; Zeman and Siakotos, 1973; Warburg, 1982).

Sea blue histiocytes have been reported to occur in the bone marrow of patients with juvenile and infantile forms of ceroid-lipofuscinosis (Smith, 1974; Gadoth, 1982; Gadoth et al., 1975; Miley et al., 1978) but this was not a universal finding.

These abnormalities have not been reported from animals with ceroid-lipofuscinosis.

#### Gross pathology

The common gross findings were a reduction in the size and weight of the brain which was more severe in the infantile form. The

loss was associated with a diminution of the cerebral gyri in all forms of the disease (Zeman, 1976). An associated cerebellar atrophy occurred in the infantile and late infantile forms but was not a constant feature in the juvenile form. A yellow-brown discolouration of the cortex and to a lesser extent the sub-cortical grey matter was also reported.

### Histopathology

The earliest report on the accumulation of intra-cytoplasmic yellow material in neurones was made by Spielmeyer (1905). These characteristic yellow-brown, periodic acid Schiff (PAS) positive, Sudan black positive, autofluorescent lipopigment granules have been reported in the nervous tissue in all forms of the disease. They were thought to be similar to the lipopigments ceroid and lipofuscin (Siakotos et al., 1970). However there has been no strong evidence that the bodies have any detrimental effect per se. The quantity of stored material, the rate at which it accumulates and the degeneration of nervous system function varied between the sub types.

The neurohistopathology of infantile disease was extensively studied and sub divided into three separate phases Haltia et al., 1973a, b; Haltia, 1982). Stage one occurred up to 2.5 years of age and consisted of a slight to moderate loss of cortical neurones and the cytoplasm of the remaining cells was distended with typical lipopigment. These changes were associated with an intense fibrillary astrocytosis in the cerebral cortex. Astrocytes also contained stored pigment and a moderate number of pigment filled macrophages occurred amongst the neurones. In stage two the same general changes were found but were more severe and associated with a dramatic loss

of myelinated nerve fibres from the white matter. Stage three occurred from four years of age. By this time the cortex was almost entirely depleted of neurones and consisted of a spongy network of astrocytes with moderate numbers of macrophages and an almost total loss of myelin from the white matter. However the sensory and motor nuclei of the brain stem were remarkably well-preserved.

In late infantile ceroid-lipofuscinosis the brains were smaller than normal and there was preservation of large numbers of neurones in the cerebral cortex, especially in the basal ganglia and brain stem (Zeman, 1976). The remaining neurones were mildly distended with typical storage bodies. A Golgi study by Braak et al., (1979) has shown that the small spiny neurones in the striatum of the cortex developed pigment filled appendages which they suggested may account for the extra pyramidal motor signs that develop during the course of the disease. The cerebellar cortex was severely affected with a reduction in Purkinje cell numbers and a loss of cells from the granular layer (Zeman et al., 1970). White matter changes consisted of degeneration of myelinated fibres and a concurrent astrocytosis.

In general the loss of neurones in the juvenile disease was much less severe than in the previous types. The neuronal cytoplasm was moderately distended with pigment and there was a marked increase in the number of pigment filled astrocytes. There may also be an accompanying loss of cells from the granular layer of the cerebellum (Zeman, 1976).

Pigment accumulation in the neurones occurred in the adult form of ceroid-lipofuscinosis but this usually had a more focal distribution (Zeman, 1976). There was also a diffuse partial loss of

Purkinje cells and granular cells from the cerebellum (Boehme et al., 1971).

Visceral accumulations of the characteristic storage material have been reported in all forms of the disease. The cells most commonly involved were the thyroid, pancreas, liver, kidney, skeletal and cardiac muscle, bone marrow, lymph nodes and skin (Haltia, 1973b; Dolman and Chang, 1972; Kristensson et al., 1965).

Ultrastructurally ceroid-lipofuscinosis was characterised by the intracytoplasmic accumulation of lipid rich electron dense material, with a variety of membranous and granular forms, in many cell types in the body. Some of these were described as curvilinear, rectilinear, fingerprint, tubular and membranous. A variety of combinations of one or more of these forms can also occur. On the basis of ultrastructural studies several authors attempted to correlate ultrastructural appearance with a particular clinical subgroup of ceroid-lipofuscinosis (Duffy et al., 1968; Gonatas et al., 1968). Whilst it is true that a particular type of body may predominate in a particular subgroup of the disease they are by no means exclusive to that group. Zeman et al., (1970) noted that curvilinear profiles predominated in the late infantile form while fingerprint profiles were more common in juvenile ceroid-lipofuscinosis. These findings were confirmed by Goebel et al., 1979 and they made the point that subgroup differences in the ultrastructure of stored lipopigments was a quantitative rather than a qualitative phenomena.

### Retinal pathology

Reports concerning the retinal pathology of ceroid-lipofuscinosis were not common and the changes that were described were well advanced and very severe. The first detailed description was that of Stock (1908) who examined eyes from three of Spielmeier's (1905) patients. He reported a primary neuro-epithelial degeneration which was not accompanied by secondary changes to the remaining retina or the optic nerve.

Reports of the retinal lesions in infantile ceroid-lipofuscinosis showed that there was severe atrophy of the retina with loss of ganglion, bipolar and visual cells. There was an accompanying proliferation of glial cells and the presence of many pigment laden macrophages. The optic nerve showed demyelination (Haltia et al., 1973b; Tarkkanen et al., 1977).

Zeman et al., (1970) examined the retina from one case of late infantile disease and found loss of the neuroepithelium but preservation of the nerve fibre layer. Sudan black staining showed that lipopigment granules accumulated in the ganglion cells and in the cells of the inner nuclear layer. A more detailed description of a single case was presented by Goebel et al., (1977) who found severe disorganization of the normal retinal architecture with complete loss of photoreceptors from all areas except for remnants in the equatorial and peripheral regions. All of the other retinal layers were normal except for the outer plexiform layer in which only a few fibres remained. The ganglion cells were often vacuolated and contained lipopigment granules. Intra-cellular melanin granules were found in all layers but were more common in the outer layers.

Findings from a less severely affected retina were reported by Schochet et al., (1980). Their patient was younger than Goebel's, as retinal atrophy had only been diagnosed 18 months prior to death. An examination of the eyecup by trans-illuminated light revealed a pigment ring surrounding the macula which was not noticed on clinical examination. The general retinal architecture was well-preserved. In the mid periphery was mild "autolysis" of rod and cone outer segments but preservation of the inner segments was good. From the periphery, the outer nuclear layer was gradually thinned and eventually disappeared at the fovea. PAS positive, autofluorescent material was observed in the ganglion cells, inner nuclear and amongst degenerate outer retinal layers in the region of the macula. The pigment epithelium underlying the macula had areas of degeneration and a loss of pigment granules from the apical cytoplasm. Melanomacrophages which had 'migrated' from the pigment epithelium were thought to be responsible for the abnormal pigment ring surrounding the fovea.

An extensive report on the retinal pathology of three cases of juvenile ceroid-lipofuscinosis was made by Goebel et al., (1974). They were similar but varied in the degree of damage that had occurred. The ganglion cells were rounded and their cytoplasm contained typical lipopigment. This material was also present in the bipolar cells of the inner nuclear layer. The most dramatic change was the almost total loss of rod and cone outer segments and the outer nuclear and plexiform layers. In one case there was a relative sparing of the peripheral cones. The remnants of the neuroretina were adherent to the underlying pigment epithelium and choroid in the other cases. Pigment epithelial cells were flattened and in the

central area had lost their melanin pigment granules. Melanin laden macrophages were commonly found in the retina especially around the retinal vasculature. There was a slight loss of myelinated axons and mild fibrillary gliosis of the optic nerve in two of the cases.

Prior to the report by Dom et al., (1979) it was thought that retinal involvement was not a feature in the adult form of ceroid-lipofuscinosis. In this case there was dilatation of the rod outer segments by yellow-brown granules. There was a lack of ganglion cells and those that remained were filled with typical auto-fluorescent lipopigments.

#### Retinal ultrastructure

Ultrastructural studies of the retina confirmed the light microscopic findings in all the sub groups of ceroid-lipofuscinosis. The stored material was similar in appearance to that found in other cells and tissues in the body and was made up of membranous, curvilinear, fingerprint profiles or granular material. Curvilinear profiles predominated in the late infantile and juvenile forms of the disease (Goebel et al., 1974, 1977; Schochet et al., 1980) whilst granular osmiophilic bodies were found in the infantile form (Tarkannen et al., 1977).

In those cases where remnants of photoreceptor cells remained these contained lipopigment typical of the particular sub-group. Cells in the inner nuclear layer also harboured typical storage bodies but the amount in a particular cell type was variable. The ganglion cells invariably contained more pigment bodies than any other cell type within the retina. Storage bodies occurred in the

pigment epithelium and this was sometimes accompanied by the loss of apical pigment granules and atrophy.

#### Canine Ceroid-lipofuscinosis

Canine ceroid-lipofuscinosis has been studied in a strain of inbred English Setters maintained in Norway (Koppang, 1970, 1973/74, 1982). Initial clinical abnormalities occurred from 14 to 18 months when "mental dullness" and reduced vision were apparent. Affected animals became stiff gaited and ataxic and convulsions occurred from 17-24 months of age and no animals have survived beyond 26 months.

In advanced cases there was atrophy of the brains so that they were reduced to approximately 70% of normal weight. The griseae were reduced in size and discoloured yellow-brown, while the brain texture was firm. There was dilatation of the lateral and fourth ventricles and an increased amount of cerebro-spinal fluid.

Light microscopy of cells of the central and peripheral nervous systems showed a gradual increase in the amount of autofluorescent lipopigment with age and virtually all neurones contained some pigment by six months of age. By 20-26 months of age neuronal cell death became obvious, especially in the cerebellar cortex. Changes in the nervous system were accompanied by a gradual increase in the amount of typical lipopigment in the viscera and other tissues in the body.

Electron microscopically, neurones from affected animals showed abnormal 'cytoplasmic condensations' from 2 days of age. These structures were less than 0.1  $\mu\text{m}$  in diameter and were granular or membranous in appearance. They were thought to be precursors of storage bodies which were modified following autophagy. They were

not thought to be associated with any particular intracellular organelles. The storage bodies were generally composed of highly organised five layered membranes which were curved and separated by a granular matrix, the so called curvilinear body. Other forms of stored material, classified as finger print and crystalloid, were also found.

In contrast to the human diseases there were no obvious ophthalmoscopic changes in the fundus of affected dogs apart from the tapetum nigrum which seemed 'paler' than normal (Koppang, 1970; 1980). Affected animals often seemed to be unable to see food and lacked a menace response (Neville et al., 1980). Electroretinograms from dogs with ceroid-lipofuscinosis showed reductions of the 'a' and 'b' wave amplitudes of between 30 and 73% (Armstrong et al., 1980; Berson and Watson, 1980). Nilsson et al. (1983), in a study using dogs which were older than those previously available, were able to show a reduction of approximately 80% in the 'b' wave amplitude. In addition there were changes to the standing potentials in the retina and the 'c' wave was replaced by a negative potential. These changes were taken as indicative of abnormalities in the pigment epithelium.

Light microscopy of affected retinas revealed a well-preserved retina with no visible abnormalities apart from intracytoplasmic granules in some ganglion cells (Koppang, 1970; 1982; Goebel et al., 1979; Neville et al., 1980). At the ultrastructural level almost every cell type in the retina contained characteristic storage bodies. The outer segments of rods and cones were of normal appearance and did not contain stored lipopigments. The pigment epithelium at all ages was filled with abnormal electron dense

material. This was commonly found to be made up of a configuration of light and dark lamellae arranged in linear or curved arrays. This material was freely dispersed in the cytoplasm or was associated with melanin granules. These pigment changes may be important in explaining the development of the electroretinographic abnormalities. In the study by Neville et al. (1980), the retinas from dogs of various ages were studied and it was recorded that the number of storage bodies per cell did not seem to increase with age.

#### Ovine Ceroid-lipofuscinosis

Ovine ceroid-lipofuscinosis was first described in an inbred flock of South Hampshire sheep by Jolly and West (1976). Affected animals showed signs of blindness and behavioural abnormalities from 11-12 months of age (Jolly et al., 1980; 1982). Motor dysfunctions commenced thereafter with episodes of muscle twitching, jaw champing and head nodding which increased in severity as the disease progressed.

Grossly, there was a reduction in the size and weight of the brain and some dilatation of the lateral ventricles. The reduction in size was due to a loss of the cerebral grey matter.

Histologically, the ovine disease was characterized by the intracytoplasmic accumulation of PAS positive, sudanophilic, autofluorescent lipopigments in the neurones and a wide variety of other cell types. There was a loss of neurones from the cerebellar cortex which also showed a mild gliosis and a marked astrocytosis. In contrast to the human and canine forms there was no marked loss of neurones from the cerebellar cortex. The occurrence of

characteristic lipopigments in the liver, kidney, heart, adrenal glands and other tissues reflected the generalized nature of the disease.

Ultrastructurally, the common lipopigment inclusion was a round or irregularly shaped body from 0.2 -5.0  $\mu\text{m}$  in size with a granular electron dense matrix in which a variety of membranous profiles occurred. These profiles were analogous to the storage bodies found in other forms of the disease namely, curvilinear, fingerprint and crystalloid.

Ophthalmoscopically, the fundus of severely affected sheep showed a slight attenuation of the retinal vasculature. No other fundal abnormalities were found.

In contrast to canine ceroid-lipofuscinosis, the retina of sheep with this disease was severely affected (Jolly *et al.*, 1980, 1982; Graydon and Jolly, 1984). Typical lipopigment granules were found in all layers of the retina except the inner and outer plexiform layers and the nerve fibre layer. The ganglion cells in particular contained much stored material. There was also a retinal atrophy which varied with the age of the animals. In its mildest form there was some degeneration of rods and cones and a loss of nuclei from the outer nuclear layer. Severely affected animals had an almost complete loss of photoreceptors with the outer nuclear layer reduced to a single row of nuclei. Ultrastructurally, typical storage bodies were found in affected retinal cells.

#### Biochemistry

The biochemical defect(s) in ceroid-lipofuscinosis remain to be elucidated. Various abnormalities have been found, none of which

have occurred consistently in all forms of the disease.

Pigments isolated from cases of this disease have chemical, histochemical and ultrastructural features similar to ceroid and lipofuscin (Zeman et al., 1969). Purified lipofuscin was a black brown pigment with a density of 1.0 -1.05 and a molecular weight of  $6 \times 10^3$  -  $7 \times 10^3$  daltons (Siakotos et al., 1973; Taubold et al., 1975). Infrared, ultraviolet, neutron magnetic resonance and fluorimetric spectra indicated that it was predominantly lipid in nature. Ceroid was a yellow pigment with a density of 1.25 -1.30. Fluorescence excitation and emission spectra of ceroid and lipofuscin were similar but not identical.

#### Peroxidase deficiency

In 1969 Chio and Tappel (1969a, b) found that Schiff bases synthesized from malonaldehyde and amino acids had fluorescence spectra similar to lipofuscin. Zeman (1974) proposed that since malonaldehyde could be produced during free radical peroxidation of polyunsaturated fatty acids, the formation of fluorescent lipopigments in ceroid-lipofuscinosis could be due to a disorder of peroxidation. This hypothesis received support when Armstrong et al., (1974) reported a deficiency of p-*para*phenylene diamine specific peroxidase from the neutrophils of some patients with ceroid-lipofuscinosis. Since the initial report further studies have indicated that this probably reflected abnormal partitioning of the enzyme between free and bound forms rather than a deficiency per se (Armstrong et al., 1982). Therapeutic trials using various antioxidants and free radical scavengers, which should protect

tissues against lipid peroxidation, failed to produce any significant clinical improvement.

#### Abnormal fatty acid profiles

An analysis of brain lipids in the infantile disease (Svennerholm, 1976) revealed very low concentrations of sphingolipids. Ganglioside levels in the cerebral cortex were reduced to 10% of the control values, while white matter cerebroside were reduced to 2% of normal values. Ethanolamine phosphoglycerides of the cerebral cortex had a much higher proportion of 18:1 and 20:4 (n-6) and much lower levels of 22:4 (n-6) and 22:6 (n-3) than controls. Svennerholm postulated that a defect in arachidonic acid metabolism leads to secondary degenerative change.

#### Retinoyl complexes

Another hypothesis for the aetiology of these diseases was proposed by Wolfe et al. (1977) who suggested that the fluorescent complexes isolated from a case of late infantile ceroid-lipofuscinosis were due to retinoids probably derived from retinoic acid. These have been further characterized (Wolfe et al., 1981) and a lipid soluble fraction containing elevated amounts of polyisopenols whilst the lipid insoluble fluorescent fraction was thought to be a retinoid compound.

#### Genetics

The ceroid-lipofuscinoses are rare diseases with a world wide distribution which have been described in all the common races and major ethnic groups and in both sexes (Zeman, 1976). Prevalence figures were difficult to estimate because of problems with the

precise diagnosis of the various subgroups. Definitive genetic studies of the infantile disease have not been carried out, but the available evidence suggested an autosomal recessive inheritance similar to late infantile, juvenile, canine and the ovine diseases (Jolly et al., 1981, 1982; Koppang, 1973/74; Zeman, 1976).

## CHAPTER 2

### EARLY DIAGNOSIS

#### Introduction

As the pathogenesis of ceroid-lipofuscinosis remains an enigma a time course study of the cellular changes in the retina was planned as a possible means of understanding the basic nature of the disease. It is necessary to detect affected animals and commence the study as early as possible in the pre-clinical stage. For this reason methods of accurate early diagnosis were investigated.

In human patients suspected of having neuronal ceroid-lipofuscinosis, a variety of criteria have been used to confirm the diagnosis. These included lymphocyte vacuolation; examination of skin, rectal, muscle, liver and brain biopsy material and cells in the urinary sediment for lipopigment bodies and the examination of bone marrow smears for 'sea blue histiocytes' (Smith, 1974; Dolman et al., 1975 and 1980; Gadoth et al., 1975 ; Goebel et al., 1975; O'Brien et al., 1975; Miley et al., 1978).

In 1977 and 1978 several of these techniques were investigated to try to establish a simple and reliable method for the early diagnosis of those sheep affected with ceroid-lipofuscinosis (Janmatt 1979). Liver, rectal and skin biopsies were performed and the diagnosis was based on the presence of autofluorescent, periodic acid Schiff (PAS) and/or Sudan black positive granules in hepatocytes, macrophages in the rectal wall and in the glandular

cells and ducts of apocrine sweat glands. Because of the ease of specimen collection and the low risk involved it had been decided that autofluorescence in the sweat glands of the skin was the method of choice for early diagnosis.

In 1978 skin biopsy examination led to the diagnosis of ceroid-lipofuscinosis in four of the 51 lambs born that year and only those four animals developed the disease. Skin biopsies from the lambs born in 1979 were examined at 2, 3 and 6 months of age and six out of 56 lambs were diagnosed as positive. This was approximately the number anticipated considering the simple autosomal recessive method of inheritance (Jolly *et al.*, 1980; 1982). However, three of these animals failed to develop signs of the disease within 2.5 years. There were no animals which were negative by skin biopsy that subsequently developed ceroid-lipofuscinosis. It was concluded that skin biopsy was not a sufficiently reliable or accurate means for establishing the diagnosis of ceroid-lipofuscinosis. Further investigations into the early preclinical diagnosis are recorded in this chapter.

## Materials and Methods

### Animals

Skin biopsies from 80 lambs from the experimental South Hampshire flock, maintained for the purpose of breeding affected animals were examined. Because it was possible that false positive lambs from 1979 may have been heterozygous for the condition, 16 obligate heterozygote lambs, obtained by mating Romney ewes with homozygous affected rams, were included. Thirty age matched but

unrelated Southdown lambs and 12 heterozygote South Hampshire/Romney Cross lambs (50% normal and 50% heterozygous) were used as controls.

#### Skin biopsy technique

Skin biopsies were taken from the axillary region because of the abundance of apocrine sweat glands found in this area. The selected area of skin was disinfected and small pieces, approximately 0.5 cm in diameter were removed using sharp scissors. The fresh biopsies were attached to microtome chucks, frozen, and 8  $\mu\text{m}$  sections were cut and mounted in glycerol for fluorescence microscopy. Some skins were sectioned at 6, 8 and 10  $\mu\text{m}$  because the degree of fluorescence could have been related to section thickness. The sections were examined on a Reichert Immunopan microscope, fitted with a quartz halogen lamp, using excitor filter 30, 8x1 FITC3 and barrier filter 18 x 3 OG 515 GG9 giving a wave length of 500 nm. In some cases a portion of the skin biopsy was fixed in 10% formalin and paraffin embedded. Paraffin embedded sections were stained by the PAS method.

#### Bone marrow biopsy technique

Bone marrow aspirates were obtained by sternal puncture using the method of Grunsell (1951). Fresh flecks of marrow were spread on a clean microscope slide, air dried and stained with McNeal's tetachrome stain. After 10 minutes the stain was diluted with slightly more than an equal amount of phosphate buffer of pH 7.2 and left on the smears for a further 10 minutes before rinsing with tap water. A drop of immersion oil was placed on the dry smears which were then coverslipped and examined under the high dry objective of the microscope.

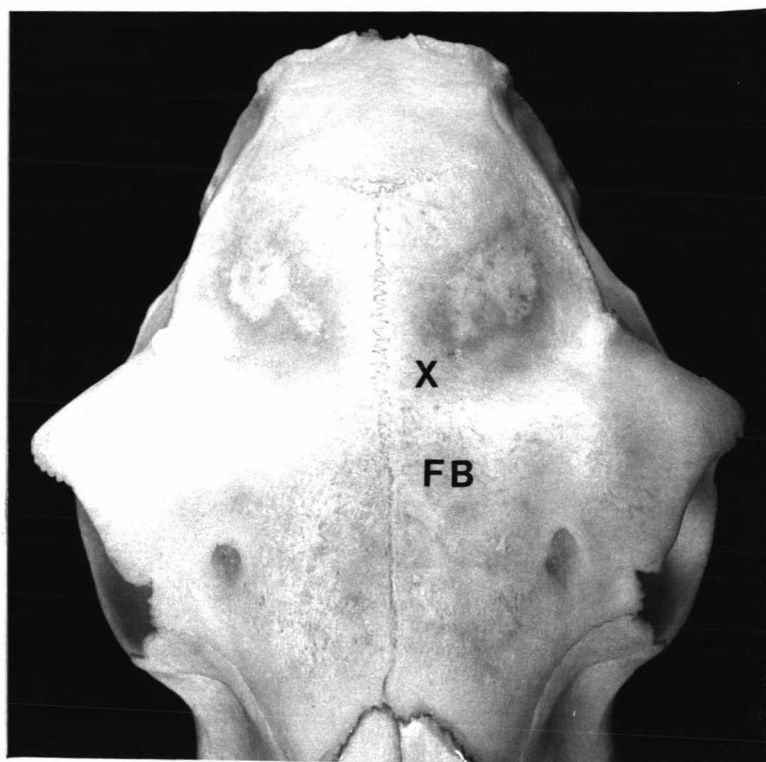
### Brain biopsy technique

Brain biopsy was performed using a modification of the technique for cattle reported by Johnston and Callow (1963). The site chosen for the biopsy was approximately 1.0 cm behind a line joining the lateral canthi of the eyes and 0.5 cm lateral to the midline (Fig. 2.1). The animals were anaesthetised with intravenous pentobarbitone sodium and anaesthesia was maintained using oxygen and halothane. The operative site was shaved and the skin prepared for surgery using aqueous and alcoholic solutions of Hibitane (ICI Tasman, Upper Hutt). The area surrounding the site was covered with drapes and the skin incised. A 2.0 mm hole was then made through the cranium with a round dental burr driven by a portable electric dental drill. The sample was obtained using a short bevelled 14 gauge needle inserted through the hole until it came in contact with the meninges. A 10 ml syringe was attached and negative pressure produced by withdrawing the plunger to the 3.0 ml mark. The needle was then pushed approximately 5.0 mm into the cortex and withdrawn. This procedure usually resulted in a core of brain tissue, some cerebrospinal fluid and occasionally blood in the syringe. If the first attempt failed to produce a sample the procedure was repeated. The skin incision was closed with a single suture.

The biopsy was fixed in 10% formol saline and paraffin sections prepared. These were stained by the PAS and Sudan black methods. Sections for fluorescent microscopy were deparaffinised and mounted in fluoromount.

All 15 animals recovered rapidly from surgery and post operative antibiotic cover was not given. Temperature, pulse and respiration rate were monitored for 5 days following surgery.

Figure 2.1: A sheep skull showing the location for brain biopsy (X). The frontal bone is indicated by (FB).



Recovery was uneventful except for one animal that developed a slight fever which was successfully treated with antibiotics.

## Results

### Skin biopsy

The skin biopsy from each animal was examined and classified into four groups based on the amount and type of autofluorescence which occurred in the sweat glands. The results are shown in TABLE 1. Examination of paraffin sections of part of the same biopsy sample stained with PAS failed to reveal any correlation between the amount of autofluorescence in the sweat glands and the PAS positive material sometimes noted in them. Varying the section thickness between 6, 8, and 10  $\mu\text{m}$  had no significant effect on the subjective assessment of fluorescence.

### Bone marrow biopsy

None of the bone marrow smears examined from animals in group one contained any 'sea blue histiocytes'.

### Brain biopsy

Brain samples from the eight South Hampshire lambs classified in group one by skin biopsy showed autofluorescent, PAS and Sudan black positive granules in their neurones. Such material was not present in normal lambs of this age. One animal from group two showed this autofluorescent lipopigment and was diagnosed as having ceroid-lipofuscinosis.

TABLE 1

	CLASSIFICATION				
	Number Examined	Abundant discrete autofluorescent granules (1)	Small number of discrete autofluorescent granules (2)	Diffuse autofluorescent non-granular (3)	No autofluorescence (4)
South Hampshire	80	8	5	61	6
Obligate Heterozygotes	16	-	4	7	5
Southdown Control	30	2	8	13	7
South Hampshire/Romney 50% heterozygous 50% normal	12	-	2	6	4
TOTALS	138	10	19	87	22

Subjective classification of autofluorescent material in the apocrine sweat glands of South Hampshire and control sheep.

### Fate of lambs

All nine lambs diagnosed as having ceroid-lipofuscinosis on brain biopsy subsequently developed the disease which was confirmed histologically following autopsy. No other lambs developed clinical signs of ovine ceroid-lipofuscinosis in the following two years.

### Discussion

The finding of three false positive animals in the lambs born in 1979 prompted speculation that this may have been due to the detection of heterozygous animals or to some environmental factor or factors. Because it was important to ensure that the correct diagnosis was made and because of the potential importance of being able to detect heterozygotes, lambs born in 1980 were examined to try and correlate skin and brain biopsies with each other and with homozygosity or heterozygosity.

Previous studies have shown that ovine ceroid-lipofuscinosis has an autosomal recessive mode of inheritance (Jolly et al., 1980; 1982). Theoretical expectations suggested that 10 affected animals would have been produced from the 80 South Hampshire lambs born in 1980, assuming that 50% of the ewe flock was heterozygous. This was in reasonable agreement with the number of animals classified as affected on the basis of the skin biopsy results. From the diagnostic point of view it was significant that one of the lambs from the class 2 classification of skin biopsies was considered to be affected when brain material was examined. Biopsy material from obligate heterozygote animals did not fall consistently into any particular category. Whilst skin biopsies were a useful method for screening large numbers of animals, the presence of small amounts of

autofluorescent material in sweat glands did not indicate heterozygosity nor rule out the diagnosis of ovine ceroid-lipofuscinosis.

Autofluorescent material in the sweat glands of control animals was not unexpected because small amounts have been reported by Janmatt (1979). However, finding large amounts of this material in the sweat glands of two control animals was significant. It was unfortunate that these animals belonged to another experimental group and were not available for further study. The source of this material remained a mystery but it was thought that it might be due to the incorporation of plant carotenoids or their fluorescent metabolites into the lipids which are secreted by the sweat glands. Because this material was found in only two animals it may also have been a function of the secretory stage at which the sweat gland was examined or of some individuals within the Southdown breed.

'Sea blue histiocytes' have been reported to occur in human patients with the juvenile and infantile forms of neuronal ceroid-lipofuscinosis (Smith, 1974; Gadoth et al., 1975 and Miley et al., 1978). South Hampshire lambs affected with ceroid-lipofuscinosis did not show 'sea blue histiocytosis' nor did they have the vacuolated lymphocytes which occurred in the juvenile form of ceroid-lipofuscinosis in man (Zeman and Siakotos, 1973; Schwendeman, 1976; Janmatt, 1979; Warburg; 1982). 'Sea blue histiocytosis' has been reported to occur in a variety of pathological conditions in man and some authors considered it to be a manifestation of the degenerating macrophage (Varela-Duran et al., 1980). This abnormality does not appear to have been reported in domestic animals.

Brain biopsy results confirmed the usefulness of this tissue in making the definitive diagnosis of ceroid-lipofuscinosis. A small amount of autofluorescent, PAS and Sudan black positive material did accumulate in the central nervous system of normal sheep as they aged (Janmatt 1979). However this occurred at a later age and was not present in the neurones of the control animals examined.

From these findings, and because false positive have not occurred, the most practicable way of making an early, definitive diagnosis of ovine ceroid-lipofuscinosis is to screen all suspect animals by brain biopsy.

## CHAPTER 3

### ELECTRORETINOGRAPHY AND OPHTHALMOSCOPY

#### Introduction

Blindness was a common finding in most forms of human ceroid-lipofuscinosis but the age of onset varied. It was the common presenting symptom in the juvenile form of the disease but usually followed mental and motor retardation in the infantile and late infantile disease. Typical ophthalmoscopic findings in all forms of human ceroid-lipofuscinosis were reported as atrophy of the optic disc and attenuation of the retinal vasculature (Zeman, 1976; Libert et al., 1982). Pigment alterations were quite variable and ranged from hypopigmentation of the peripheral fundus, dystrophy and brown discolouration of the macula in infantile cases (Santavouri et al., 1973; Santavouri, 1982) to perimacular hypopigmentation and mottling of the macula in some cases of juvenile and late infantile disease (Goebel et al., 1977; Schochet et al., 1980; Spalton et al., 1980; Warburg, 1982; Zeman and Siakotos, 1973). In canine ceroid-lipofuscinosis blindness occurred very late in the disease and pigment abnormalities have not been recorded although Koppang (1982) stated that the tapetum nigrum seemed 'paler' than normal. Sheep with ovine ceroid-lipofuscinosis had blindness as the initial clinical abnormality and as such resembled the juvenile form of the human disease. Ophthalmoscopic examination of the fundus of advanced

cases revealed slight attenuation of the retinal vessels but no pigment abnormalities (Jolly et al., 1980; 1982).

The electroretinogram (ERG) is the summation of action potentials generated by the cells which make up the various layers of the retina, following stimulation by light. This response has been known for over 100 years and the terminology used to describe the components of the ERG was introduced by Einthoven and Jolly in 1908. They designated the first negative deflection the 'a' wave which was followed by a larger positive 'b' wave and another slower positive deflection known as the 'c' wave. In recent times improved techniques of recording and analysis have enabled more components to be discovered but these are beyond the scope of this chapter and are not considered further. The ERG is usually recorded using a corneal contact lens electrode which reduces the level of background noise and interference and this, coupled with signal averaging systems, has led to significant improvements in electroretinographic technique. However these techniques are not standard and there is difficulty comparing results from different laboratories.

The contribution each type of retinal cell makes to the ERG was not fully understood. Microelectrode and selective toxicity studies indicated that the 'a' wave was produced by the photoreceptor cells, probably the inner segments. The Muller or bipolar cells were suggested as the site of origin of the 'b' wave. Retinal pigment epithelial cells have been shown to be the site of origin of the 'c' wave (Galloway, 1975; Knave et al., 1972; Tomita and Yanagada, 1981).

The size and shape of the ERG depends on a number of physiological variables such as the wavelength, intensity, duration,

and frequency of the light stimulus as well as the state of retinal adaptation, eye position and the state of consciousness of the subject.

The ERG has been used in many clinical investigations of patients with various forms of ceroid-lipofuscinosis and these have been considered in more detail in Chapter 1. Most reports in the human literature showed a completely absent or severely depressed ERG which reflected the late stage in the disease process at which recordings were made (Harden et al., 1973; Harden and Pampiglione, 1982; Pampiglione and Harden, 1977; Tackmann and Kuhlendahl, 1979). Those cases where repeated ERG have been done on the same patients a progressive decline in the amplitude of the response was revealed (Goebel et al., 1977; Tarkkanen et al., 1977).

Berson and Watson (1980) have reported ERG findings from English Setter dogs with ceroid-lipofuscinosis and these animals had a 30-40% reduction in 'b' wave amplitude. This small reduction was not unexpected because pathological changes to the retina of these dogs was minimal even at the end stage of the disease process (Goebel et al., 1979; Neville et al., 1980). A more significant reduction was reported by Armstrong et al. (1982) who found that in 24 month old dogs the 'a' wave was reduced by 50% while the 'b' wave amplitude had declined by 63%. Nilsson et al. (1983) reported a severe decline in 'a' and 'b' wave amplitudes and change from a positive to a negative 'c' wave in severely affected dogs. The change to the 'c' wave was reported to correlate with damage to the pigment epithelium.

In this chapter sequential changes in the electroretinograms of sheep affected with ceroid-lipofuscinosis are recorded.

## Materials and Methods

### Animals

Electroretinograms were recorded from seven affected South Hampshire sheep and seven sex and age matched but unrelated control animals.

### Corneal contact lens electrode

A corneal contact lens electrode was fabricated from 2.0 mm clear methacrylate sheet using a modification of a technique described by Wietzel et al., (1976). A soft impression mould of the sheep cornea was made with a silicone rubber dental moulding compound. A hard mould was then made using dental stone. Provision was made for an electrode to be incorporated in the contact lens by glueing a ring of wire 5.0 mm in diameter to the surface of the mould. A suitably sized piece of 2.0 mm clear methacrylate sheet was gently heated in a bunsen flame until it was soft and pliable. It was then draped over the hard mould and compressed using an hydraulic press with a 2.0 cm rubber bung between the press and the methacrylate to facilitate moulding. The excess material was trimmed and the edges of the lens ground to a suitable size. An eyelid speculum was manufactured in a similar manner but the central portion of it was removed before it was glued upside down to the contact lens. An electrode was made from 0.2 mm silver wire shaped to fit the depression in the lens and glued in place with cyanoacrylate cement. The free end of the electrode was passed through a small hole and soldered to a flat piece of silver which had been attached to the edge of the eye lid speculum. The leads to the

amplifier were attached to this piece of silver. A guard electrode was made by placing a circle of silver wire in the groove between the lens and the eye lid speculum to reduce the 50 Hz interference in the system.

### Equipment

Photostimulation for the ERG was provided by a Grass PS22 photic stimulator (Grass Instrument Co., Quincy, Mass., U.S.A.) which gave a flash of 10  $\mu$  sec duration. The flash intensity could be varied in five steps with ratios of 1, 2, 4, 8 and 16 with a maximal flash intensity of  $1.5 \times 10$  candle power at intensity setting 16. The intensity of the photostimulation was varied, with a relative intensity setting of '2' being used to produce a weak stimulus and '8' being a strong stimulus. The wavelength of the stimulating light was modified by placing coloured plastic filters over the face of the flash lamp.

The ERG signal was amplified using a Grass RPS 1078 power amplifier and a Grass P511E pre amplifier with a band width of 0.3 Hz to 1 kHz. This signal was then passed through a Neurolog NL 750 signal averager (Digitimer Ltd., Welwyn Garden City, Herts., U.K.). For signal averaging, a sweep time of 250 milliseconds was used which was triggered by the photostimulator. The resulting ERG was displayed on a storage oscilloscope (Tektronic Type 564, Tektronix Inc., Portland, Oregon, U.S.A.) and a Polaroid photograph provided a permanent record of the response. Thirty two separate responses were averaged to obtain the final electroretinogram at an amplification of 2000 times.

### Testing procedure

The sheep's pupil was dilated with 1% cyclopentolate and 10% phenylephrine chloride ophthalmic drops. The animal was then anaesthetized with intravenous pentobarbitone sodium, intubated and maintained with oxygen and 1-2% halothane. An effort was made to ensure that each animal was at approximately the same depth of anaesthesia during the test procedure and recordings were discontinued if there was any change. The contact lens was positioned over the cornea and a methyl cellulose, sodium chloride solution placed in the eye to form a salt bridge between the cornea and the recording electrode. A reference needle electrode was inserted under the skin on the bridge of the nose midway between the eyes. A ground electrode was attached to the brass ear tag. The discharge tube of the photostimulator was positioned 30 cm from the cornea and at 90 degrees to the centre of the cornea on the visual axis. Recordings were made from the left eye which was used on each occasion until surgical enucleation was performed after which the right eye was tested. Occasionally both eyes were tested on the same day.

Photopic electroretinograms were recorded using white light at an intensity setting of 8 and a flash frequency of 2 per second. The sheep was then allowed to dark adapt and after 20 minutes was tested with blue and then white light at an intensity setting 2 and a frequency of 1 flash per second. Following this the animal was allowed to recover from the anaesthetic or anaesthesia was maintained for surgical enucleation of the eye for histopathological examination.

### Measuring the electroretinogram

Measurements taken from the electroretinograms conformed to the conventions outlined in the introduction. The 'a' wave amplitude was measured from the base line to the lowest point and the 'b' wave was measured from that point to the highest point of the upward deflection. As well as these amplitudes, temporal aspects of the response were measured. These were the latency, which is the interval from stimulus onset to the beginning of the 'a' or 'b' wave; the implicit time which is the time from stimulus onset to the peak of the 'a' or 'b' wave and the duration of the response.

## Results

### Clinical observations

Pupillary light reflex testing and ophthalmoscopic examinations of the retina of each animal prior to electroretinography failed to reveal any abnormalities except for one animal which had slight attenuation of the retinal vasculature. Pigment changes similar to those reported in the retina in human ceroid-lipofuscinosis were not noted during fundoscopic examination of the sheep retina. Affected animals showed noticeable visual impairment from 40 to 52 weeks of age. They were slow to observe and retreat from approaching humans and had difficulty seeing objects placed in their way. They were difficult to move, grazed away from the rest of the flock and it was possible to approach and catch them in the middle of the paddock.

### Electroretinography

Typical cone photoreceptor dominated, light adapted, ERG's from sheep with ceroid-lipofuscinosis at 42, 54 and 66 weeks of age are

shown in Figure 3.1 and these reveal a decline in 'a' and 'b' wave amplitudes with increasing age. At the end of the experimental period a recognisable ERG could still be recorded although at very much reduced amplitude (Fig. 3.1c). The b wave amplitude relative to age were plotted and were in the low normal range at 20 weeks of age. They fell below normal soon after and then remained stationary until the sheep were 52-56 weeks of age before gradually declining (Fig 3.2). Plots of the a wave amplitudes were more variable but these also had a general downward trend with increasing age.

Dark adapted, rod dominated, responses from an affected sheep at various ages are shown in Figure 3.3 and they reveal a decline in 'a' and 'b' wave amplitudes with increasing age. However, unlike the cone responses the ERG was virtually extinguished when the animal was 62 weeks of age (Fig. 3.3c). The 'b' wave amplitudes relative to age were plotted and declined more rapidly and at an earlier age than the cone responses (Fig.3.4). The 'a' wave amplitudes were also plotted relative to age and these were more variable but there was a general downward trend. Plots of dark adapted 'a' and 'b' wave responses from individual sheep at various ages showed some variability but the overall trend was a continual decline from soon after testing was commenced (Fig 3.5).

By the test methods used in these experiments there were no apparent changes to the temporal aspects of the electroretinogram.

#### Discussion

Electroretinographic testing of sheep with ceroid-lipofuscinosis revealed that the decline in visual function began at approximately 6 to 7 months of age and occurred several months before the development

Figure 3.1: A series of cone dominated electroretinograms from a sheep with ceroid-lipofuscinosis at (A) 42 weeks of age (b) 54 weeks of age (c) 66 weeks of age. Note that at 66 weeks of age an ERG is recordable from the animal.

Figure 3.2: Cone dominated, light adapted, 'b' wave amplitudes of normal lambs ( $\square$ ) and in lambs with ceroid-lipofuscinosis ( $\bullet$ ) relative to age. A typical cone dominated ERG is shown in the inset.

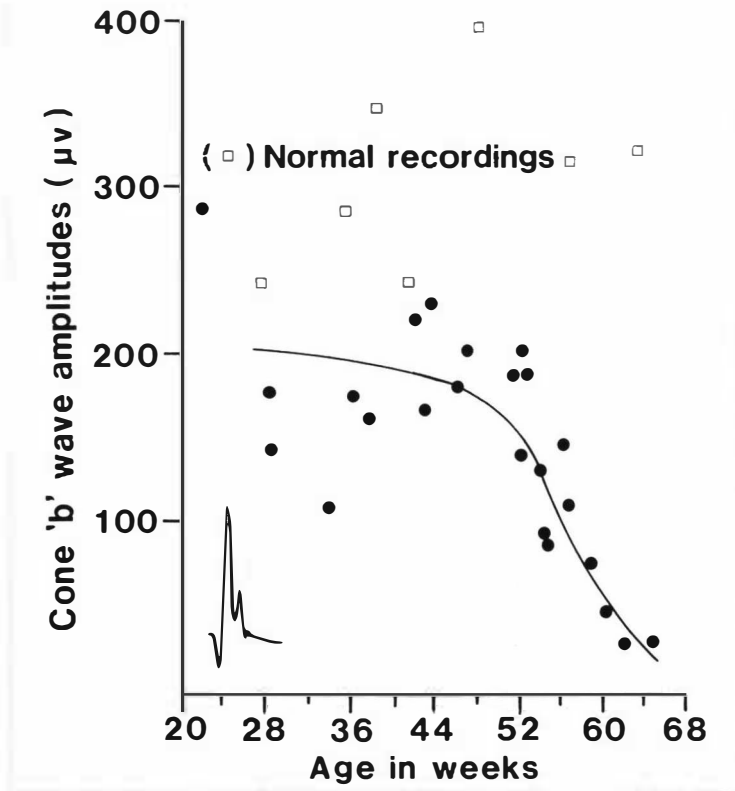
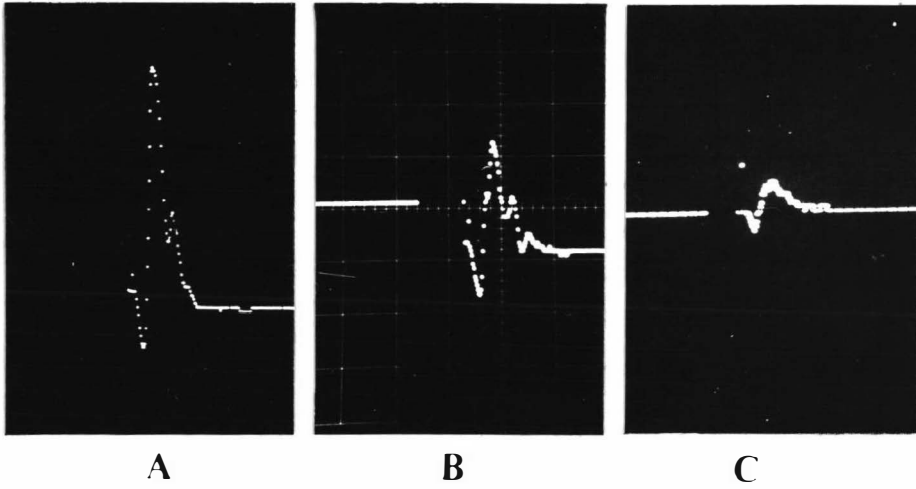


Figure 3.3: A series of rod dominated electroretinograms from a sheep with ceroid-lipofuscinosis at (A) 30 weeks of age (B) 54 weeks of age (C) 62 weeks of age. Note that compared to 3.2(C) the response is almost extinguished.

Figure 3.4: Rod dominated, dark adapted, 'b' wave amplitudes from normal lambs (■) and in lambs with ceroid-lipofuscinosis (●) relative to age and the number of nuclei (○) in the outer nuclear layer. A typical rod dominated ERG is shown in the inset.

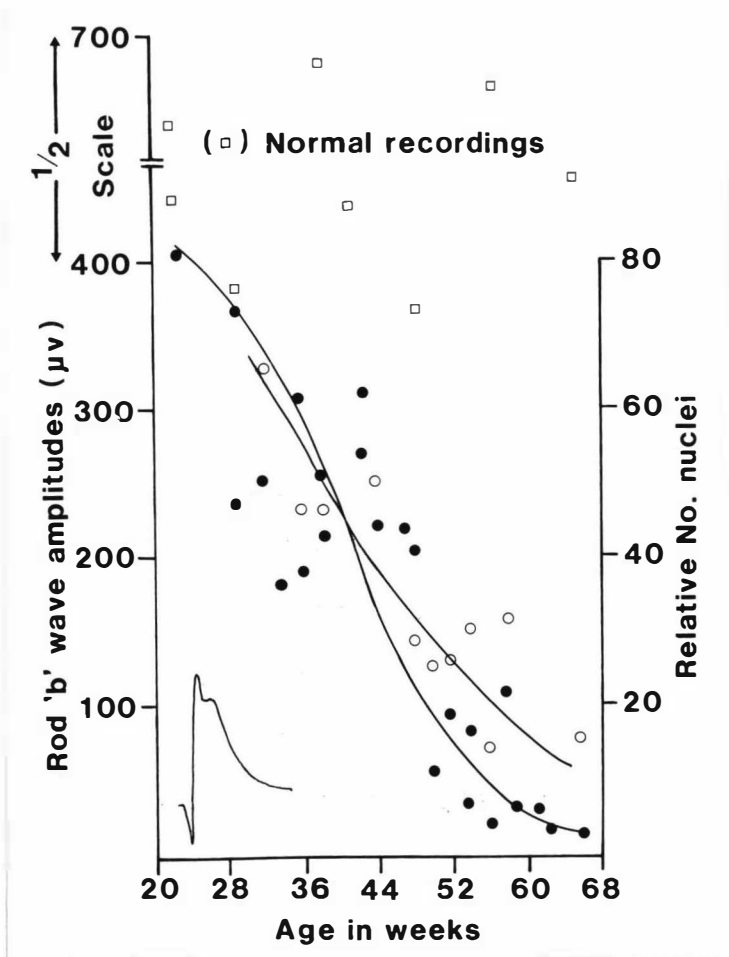
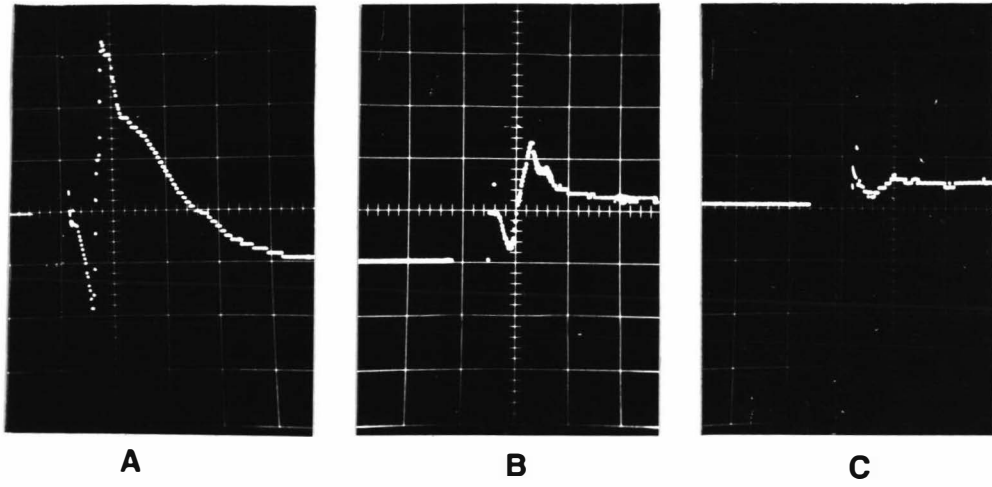
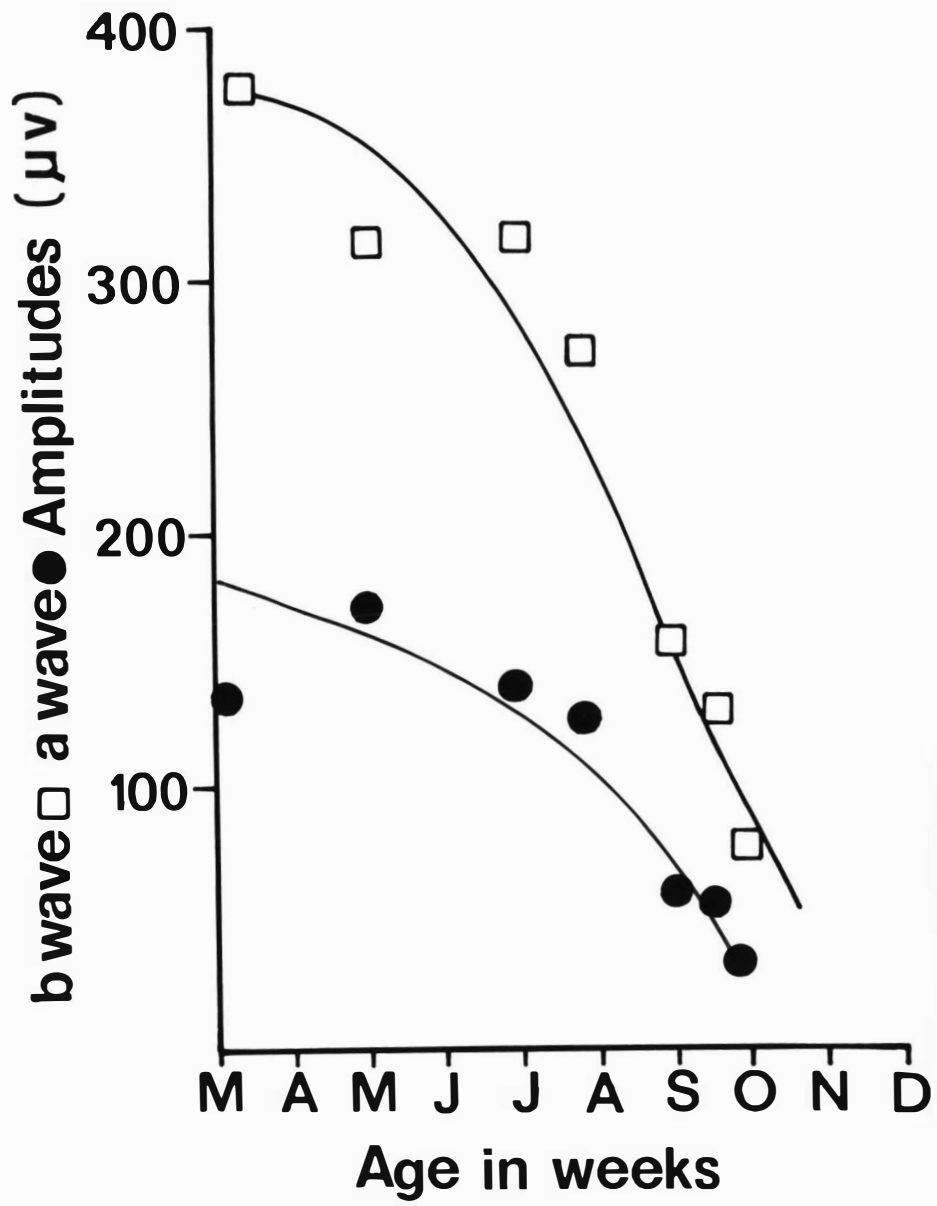


Figure 3.5: A and b wave amplitudes plotted from sequential electroretinograms from a South Hampshire sheep with ceroid-lipofuscinosis from 24-54 weeks of age.



of clinical signs and before histopathological changes were obvious in the retina.

The retina of the sheep is of the mixed type containing both rods and cones but with rod photoreceptors predominating and as such gives ERG responses typical of this type of retina (Nilsson *et al.*, 1973). Although there were marked differences in techniques the overall appearance of the responses obtained in this study were similar to those reported by Knave *et al.*, (1972). These authors also showed that dark adaptation enhanced the rod photoreceptors domination of the response whilst cone photoreceptors were dominant in the smaller amplitude, light adapted electroretinograms.

This study revealed a precipitous decline in rod responses, especially the b waves, with increasing age. Changes to the rod photoreceptor dominated ERG's were more severe and occurred at an earlier age than that of cones. These findings are in agreement with the histopathological changes reported in Chapter 4 which indicated a relative sparing of cone photoreceptor cells until late in the disease process. Rod photoreceptor responses lack the plateau from approximately 28-50 weeks of age which is a feature of the cone 'b' waves. The 'b' wave responses have been highlighted in this study because they are thought to arise from the level of Muller or bipolar cells and as such the responses have undergone a considerable degree of amplification and integration. Therefore, changes to the b wave amplitudes are a reflection of abnormalities from the level of the outer segments to the inner nuclear layer.

During this study a variety of other testing regimes, using light of differing colour and intensity were tried but none of these

yielded results which were superior to those reported in this chapter. Considerable effort was devoted to trying to develop 'c' wave recordings but these were largely unsuccessful.

Plots of 'a' and 'b' wave amplitudes from individual animals were made and although there was sometimes variability between individual animals or between individual recordings there was generally a rapid decline in ERG amplitudes over a short period of time. It would therefore be feasible to use sequential electroretinography to monitor the course of the retinal degeneration and this may be useful in assessing possible therapeutic regimes.

## CHAPTER 4

## RETINAL PATHOLOGY

## Introduction

Visual impairment occurred in all forms of ceroid-lipofuscinosis with the possible exception of the adult or Kufs type. However the retinal pathology has only been described from patients who have died with terminal disease and hence had advanced retinal degeneration with an almost complete loss of rod and cone photoreceptor cells. In these cases changes in the remaining retina were minimal apart from accumulations of characteristic lipopigment. Ganglion cells, in particular, contained the largest amount of pigment (Goebel et al., 1974; 1977; Schochet et al., 1980; Tarkannen et al., 1977).

Ultrastructural studies confirmed the light microscopic findings. Storage bodies were noted in all retinal layers which remained including the pigment epithelium. In the late infantile and juvenile forms of the disease, these were largely made up of membranous, curvilinear and finger print profiles (Goebel et al., 1974; 1977; Schochet et al., 1980) and of granular osmiophilic material in the infantile disease (Tarkannen et al., 1977).

In the English Setter canine model, reduced vision occurred only late in the course of the disease (Koppang, 1973/74; 1982). There was however, no obvious loss of, or damage to photoreceptors or pigment epithelium, even though these cells harboured massive amounts

of autofluorescent lipopigment (Neville et al., 1980; Goebel et al., 1979). The pigment epithelium sometimes contained peculiar circular, semicircular and straight stacks of membrane which were not always surrounded by a limiting membrane (Neville et al., 1980; Nilsson et al., 1983).

In ovine ceroid-lipofuscinosis, blindness was severe with an almost complete loss of most rod and cone photoreceptor cells by the time the disease was terminal (Jolly and West, 1976; Jolly et al., 1980; 1982). The present study involved a time course examination of the development of retinal pathology in the ovine form of the disease.

## Materials and Methods

### Animals

Fifteen retinas from nine affected South Hampshire lambs were examined in this study. The diagnosis of ceroid-lipofuscinosis was made by histopathological examination of brain biopsies or was confirmed by histopathology following euthanasia. Control retinas were obtained from six unrelated animals matched for sex and age.

### Experimental design

To increase the range of ages at which retinas could be examined the left eyes from six affected lambs were removed under halothane general anaesthesia using standard surgical procedures. This was usually carried out immediately after the animal was killed with an overdose of sodium pentobarbitone. Retinas were thus available for study at various ages from 26 to 84 weeks. Control retinas were collected for study at ages ranging from 26 to 66 weeks.

### Preparation of retinas for microscopy

The anterior segment of each enucleated eye was removed by a razor blade incision just posterior to the corneo-scleral junction. The vitreous humor was carefully removed and the posterior eye cup was fixed overnight in a mixture of cold 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate or cacodylate buffer of pH 7.2. Following primary fixation, the eyecup was washed three times with appropriate buffer. Small pieces of retina and the underlying choroid and sclera were obtained using a 2.0 mm biopsy punch from areas adjacent to the optic disc, mid and peripheral regions. Samples were taken from the tapetal and non tapetal portions of the nasal and temporal segments of the retina. These tissues were post fixed for 2 hrs. in 1% osmium tetroxide in 0.1 M phosphate or cacodylate buffer before dehydration in graded alcohols and propylene oxide and embedding in epoxy resin (Durcupam-ACM, Fluka, Switzerland). Sections for light microscopy were cut at 0.5 - 1.0  $\mu\text{m}$  and stained with 1% toluidine blue for 45 sec on a hot plate at 80°C. Appropriate areas for thin sectioning were cut at 70 nm and stained with saturated uranyl acetate and 1% lead citrate and examined in a Philips EM200 electron microscope.

The eyecup was divided in an anterior-posterior direction through the optic disc and the two halves were embedded in paraffin wax by routine methods. Sections were prepared and stained by periodic acid-Schiff (PAS) and Sudan black methods. Unstained deparaffinised sections for fluorescent microscopy were examined with a Reichert Immunopan microscope under blue light of 500 nm wave length.

### Cell counting

The numbers of cells present in the outer nuclear layer were estimated by counting the nuclei visible over a standard length provided by an eyepiece graticule. Counts were carried out on plastic embedded, toluidene blue stained sections taken immediately adjacent to the optic disc in the tapetal, and occasionally, the non tapetal retina. The cells were counted from three separate lengths on three separate occasions and the resulting counts were averaged. Counts were not made unless vertically orientated sections were available. An estimate of the numbers of cells present in the inner nuclear layer was obtained by counting the number of rows of nuclei.

## Results

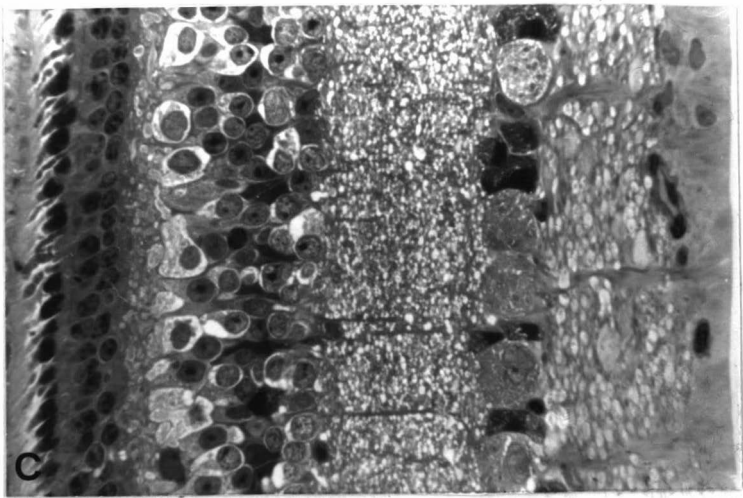
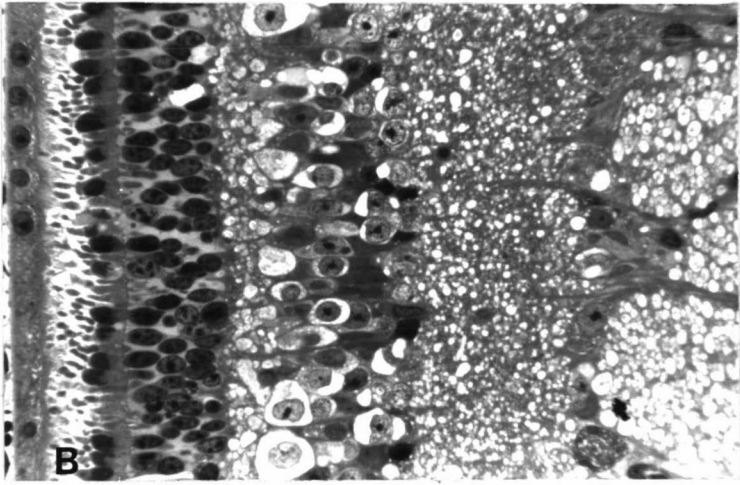
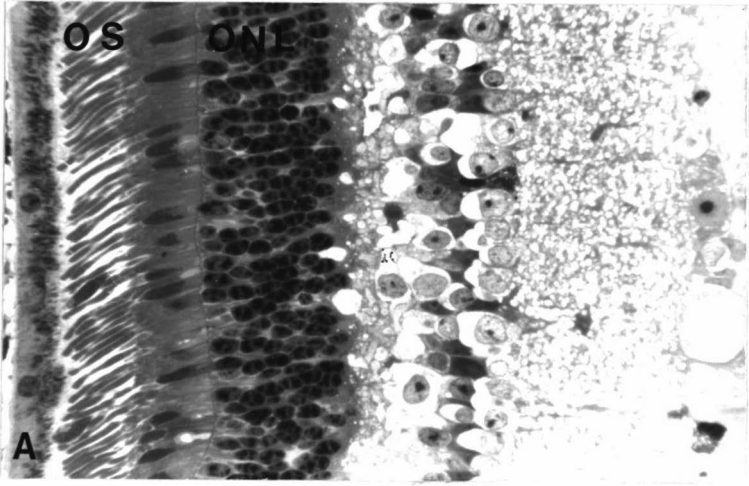
### Gross pathology

There were no alterations to the retina that were visible on gross examination of the transected eyecup.

### Histopathology

From approximately 26 weeks of age (the youngest sheep examined) storage bodies were always present in all retinal layers but were particularly common in the ganglion cells. Interpretative assessment of paraffin and epoxy embedded sections revealed a noticeable and progressive loss of nuclei from the outer nuclear layer and shortening and loss of outer segments which occurred from 36 weeks of age (Fig. 4.1). The numbers of nuclei present in the outer nuclear layer at various ages and the numbers of cone inner segments present are shown in Figure 3.4. Cone photoreceptor cells were apparently more resistant to damage and persisted for longer than rod receptor

Figure 4.1: Light micrographs of (A) a normal lamb retina (B) a lamb with ceroid-lipofuscinosis aged 48 weeks (C) a lamb with ceroid-lipofuscinosis at 66 weeks of age. There is a progressive loss of nuclei from the outer nuclear layer (ONL) and shortening and/or partial collapse of the outer segments (OS). Cone inner segments are darker than those of the rods. (Epoxy resin, toluidine blue x450)



cells but with increasing age the inner segments became broader, shorter and more rounded in outline (Fig. 4.1b,c). By the time the animals were 84 weeks of age the outer nuclear layer was reduced to a single row of nuclei which lay adjacent to the pigment epithelium. A few outer and inner segments that were thought to be those of cone receptor cells were present. Cell numbers in the inner nuclear and ganglion cell layers remained constant throughout the study period.

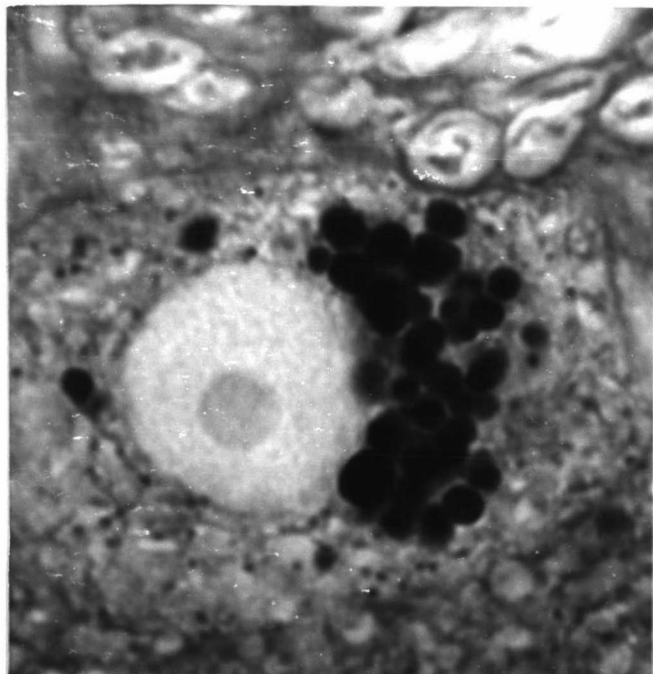
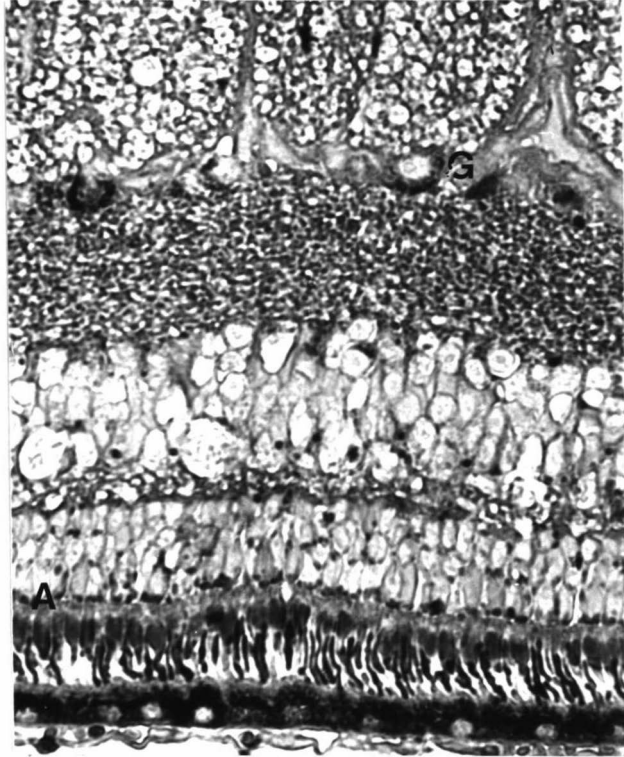
A feature of all affected retinas was the occurrence of PAS positive, sudanophilic, autofluorescent granules in a wide variety of cell types within the retina from 26 weeks of age. Storage bodies were visible in the non-pigmented tapetal area and in the rest of the pigment epithelium when this was pretreated with hydrogen peroxide to bleach the melanin granules. These were smaller in size and fewer in number than elsewhere in the retina. Smaller numbers of similar granules with similar staining properties were seen in the pigment epithelium of normal animals. Lipopigment bodies were scattered throughout the inner and outer nuclear layers and formed a band of sudanophilic material in the rod and cone outer fibres adjacent to the outer limiting membrane (Fig. 4.2). From 26 weeks of age onwards, almost every ganglion cell contained pigment granules (Fig. 4.3). A small number of smaller sudanophilic, PAS positive granules were seen in the ganglion cells of control animals. Storage bodies in all layers of the neural retina became larger and more complex with increasing age.

#### Electron microscopy

All the normal retinal layers could be identified at all stages in the disease process and the electron microscope findings confirmed

Figure 4.2: Photomicrograph of a retina from an affected sheep showing Sudan black positive granules in the ganglion cells (G) and in the inner nuclear and outer nuclear layers. Notice the distinct band of granules adjacent to the outer limiting membrane (A). The sudanophilic granules in the pigment epithelium are mainly melanin granules. (Paraffin section, Sudan black x600)

Figure 4.3: A ganglion cell from a 66 week old sheep with ceroid-lipofuscinosis showing numerous large storage bodies in the cytoplasm. (Paraffin section, Sudan black x1200)



and expanded the light microscope observations. Lipopigment bodies were found in all retinal layers but were uncommon in the inner and outer plexiform layers and the nerve fibre layer. Typical lipopigment bodies consisted of round, oval or irregularly shaped amorphous electron dense material which contained variable amounts of membranous components. They varied considerably in size and were frequently found to be surrounded by a membrane or appeared to be non membrane bound. Some of the larger accumulations appeared to be aggregations of smaller storage bodies (Fig. 4.4). The common internal structure consisted of whorls and stacks of electron dense membranes which were arranged in so called 'membranous arrays' (Fig. 4.5). Many cells, especially bipolar cells in the inner nuclear layer, contained very small electron dense areas in their cytoplasm. When these were examined at very high magnifications they were found to contain pale granular electron dense material in which were embedded darker faintly membranous structures of variable length (Fig. 4.6).

Some cells in the inner nuclear layer contained coarse membranous skeins of material dissimilar to the usual storage bodies which intertwined through the cytoplasm of the cell. In some of the same cells dilatation of the Golgi apparatus and the appearance of large numbers of small lysosome like vesicles also occurred (Fig. 4.7).

The pigment epithelium remained almost normal throughout the disease process but the apical villi appeared to become shortened following the loss of outer segment material (Fig. 4.8, 4.13). There was no hypertrophy or alterations to the basal infoldings that have been reported in some human cases of ceroid-lipofuscinosis.

Figure 4.4: A bipolar cell showing membranous accumulations which appear to coalesce to form larger storage bodies. (EM x5000)



Figure 4.5: A bipolar cell showing 'membranous arrays' which are typical of those found in storage bodies in the retina in ovine ceroid-lipofuscinosis. (EM x64,100)

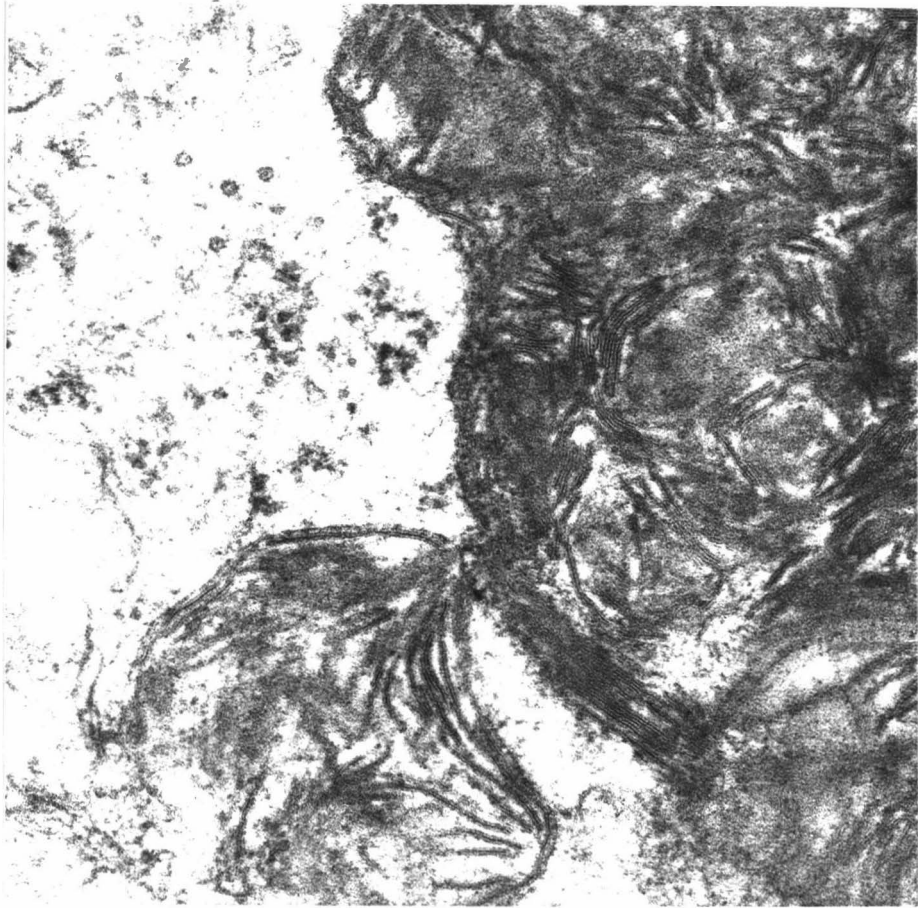


Figure 4.6: A high power electron micrograph of a small electron dense 'smudge' in the cytoplasm of a bipolar cell which shows small membranous structures (M) which are thought to be developing storage bodies. (EM x221,400)

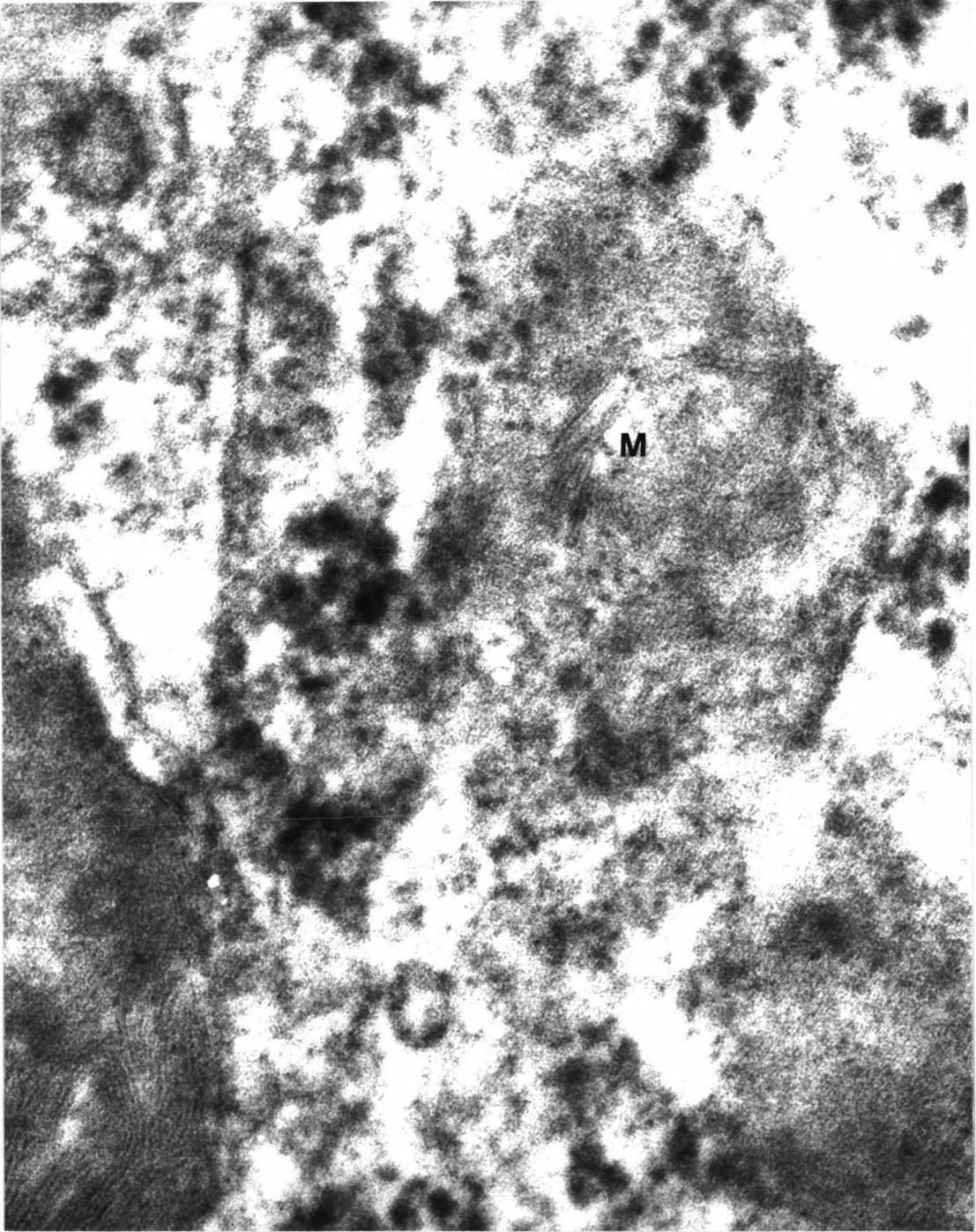


Figure 4.7: The outer fibres of rod photoreceptors showing skeins of membranous material (M) in the cytoplasm which is dissimilar to typical lipopigment bodies (L) in an adjacent cell which appear to be membrane bound. There are also large numbers of lysosome-like vesicles. There is dilatation of the golgi apparatus (G) but not the mitochondria (MT) which suggests that this is not a fixation artifact.  
(EM x25,800)



Many membranous structures could be found in all portions of the pigment epithelium and care was required to differentiate true storage bodies from phagocytosed and partially digested outer segment material. The cytoplasm of these cells in all sheep was found to be quite membranous which further complicated interpretation. Typical storage bodies were found in the pericytes in the choriocapillaris (Fig. 4.8).

From 26 weeks of age storage bodies could be found in all parts of the rod photoreceptor cells except the outer segments and were most numerous in the rod outer fibres adjacent to the outer limiting membrane. As was noted by light microscopy there was a decline in the number of nuclei in the outer nuclear layer from 36 weeks of age and the spaces formerly occupied by the nuclei and their processes were filled with pale amorphous granular material without many organelles which was interpreted as originating from the supportive Muller cells.

The earliest and most significant change noted was the formation of dystrophic rod outer segments which were kinked and irregularly formed (Fig. 4.9). There was also disruption and vesiculation of the outer segment membranes (Fig. 4.10). In the associated inner segment there was some mild dilatation of the endoplasmic reticulum and Golgi apparatus and some swelling and disruption of the mitochondria.

Cones, on the other hand, were relatively resistant to damage and even after severe changes to their outer segments their inner segments were of relatively normal appearance and remained so until late in the disease process (Fig. 4.11, 4.12). Storage bodies were present in all portions of the cone cells except the outer segments.

Figure 4.8: The pigment epithelium from a sheep with ceroid-lipofuscinosis showing shortened apical villi (V) and a typical storage body (S) in a pericyte. (EM x6000)

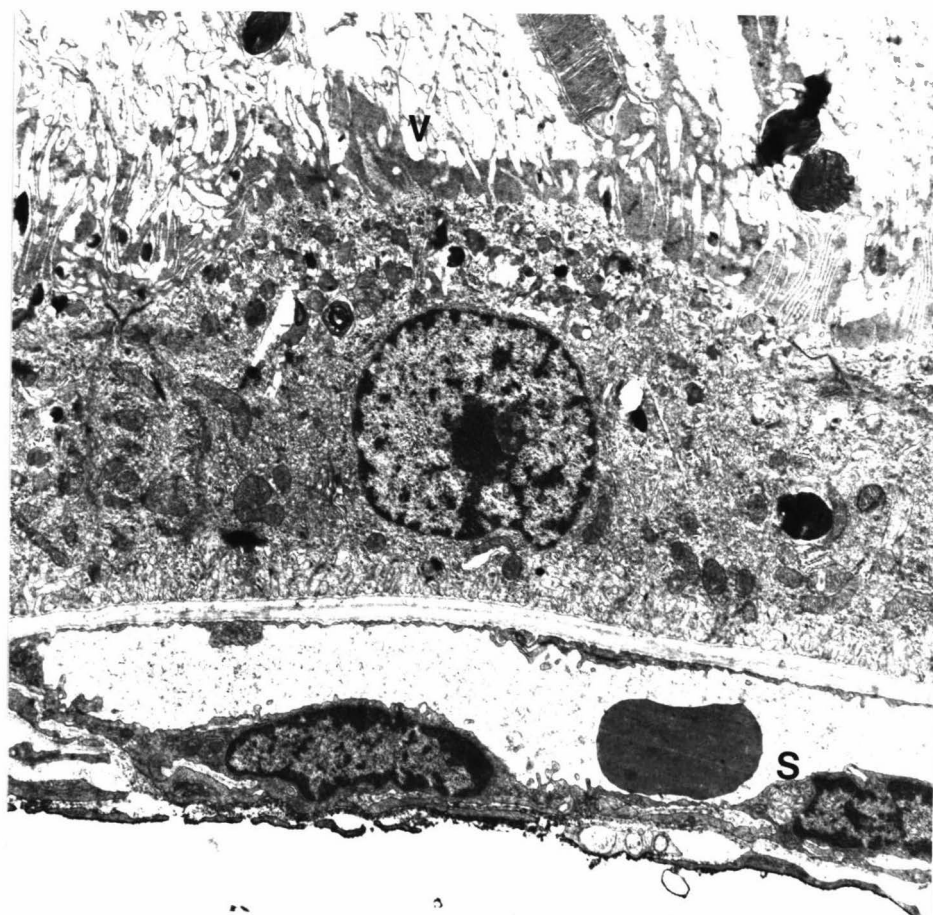
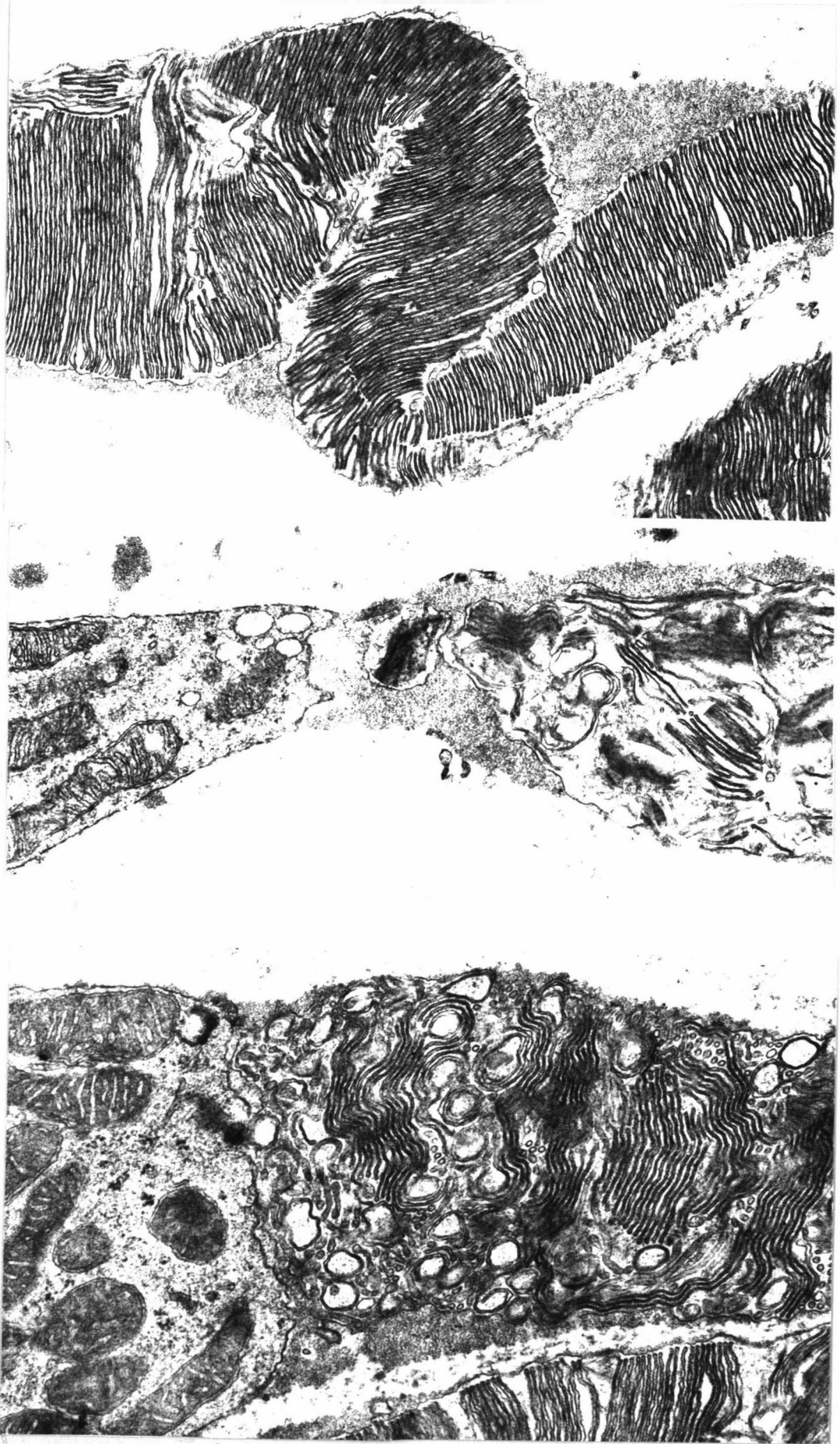


Figure 4.9: Electron micrograph of a kinked and abnormal rod outersegment. (EM x26,000)

Figure 4.10: A dystrophic rod outersegment. (EM x24,000)

Figure 4.11: Electronmicrograph of dystrophic cone outer segments. (EM x34,000)



At 84 weeks the outer nuclear layer was reduced to a single row of nuclei lying adjacent to the pigment epithelium. In the space between these layers there were remnants of outer and inner segments which were probably cones. Some mitochondria typical of those found in cone inner segments lay adjacent to the nuclei in the outer nucleic layer. Many of the remaining nuclei were pyknotic or their cytoplasm was filled with storage bodies, vacuoles and other cellular debris (Fig. 4.13).

In the inner nuclear layer, membranous arrays typical of ceroid-lipofuscinosis were found in all cell types including the Muller cells (Figs. 4.4, 4.5). These were found in the youngest animal examined and increased in number with increasing age until at 84 weeks large numbers were present in the cytoplasm of most of the cells that remained. The size and complexity of the bodies was variable and ranged from small solitary accumulations to large complex structures. Some cells also contained a few small membrane bound granular bodies which on high magnification sometimes contained membranous elements. These were not ceroid-lipofuscinosis storage bodies and were found in normal animals.

Ganglion cells usually contained the largest number of storage bodies and large numbers were present in the youngest animals examined (Fig. 4.14). This type of cell contained two types of pigment bodies consisting of membranous material (Fig. 4.14 inset) and electron dense material containing numerous tubular structures (Fig. 4.15). The latter were similar to the tubular arrays reported in neurones in the central nervous system (Jolly *et al.*, 1980).

Figure 4.12: Two abnormal cone outer segments (C) surrounding a rod (?) outer segment of normal appearance. The cone inner segment appears normal. (EM x16,500)

Figure 4.13: An electronmicrograph of the retina from a sheep with advanced retinal degeneration. The outer nuclear layer is reduced to a row of nuclei which contains a pyknotic nucleus (PN). There are remnants of inner segments (IS) and perhaps outer segments (OS) lying adjacent to the pigment epithelium (PE). The pigment epithelium appears to lack apical villi. (EM x 12,500)

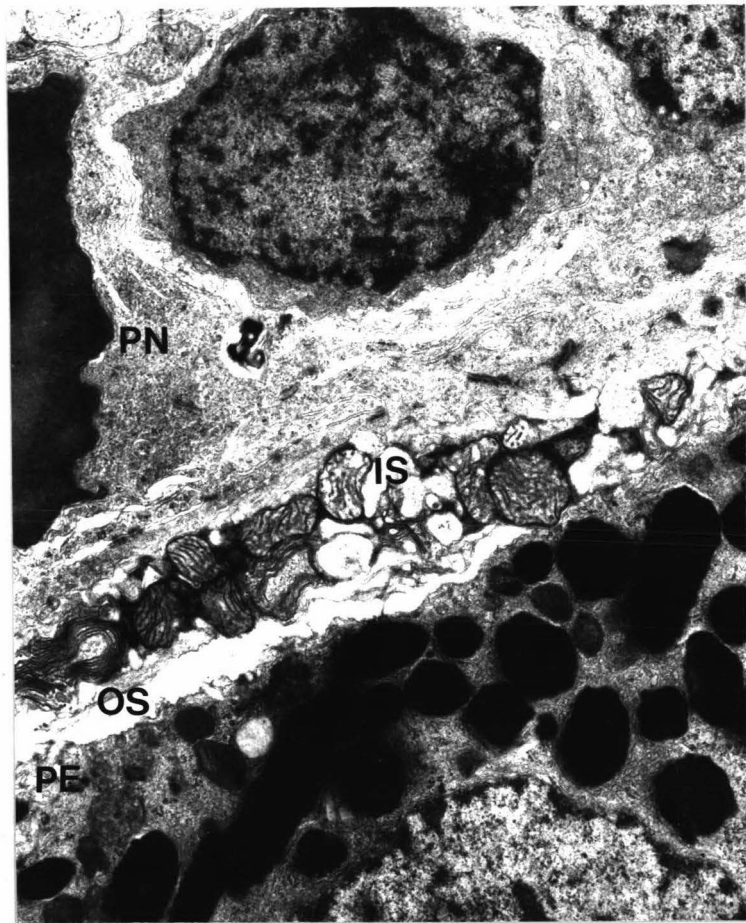
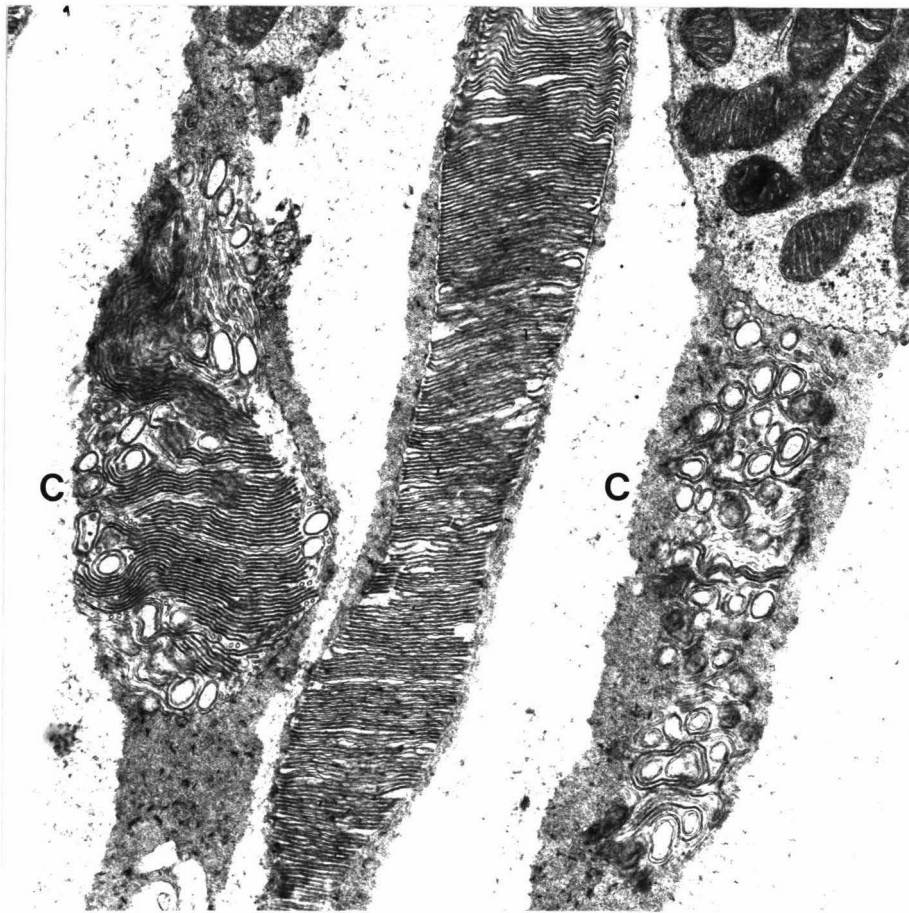
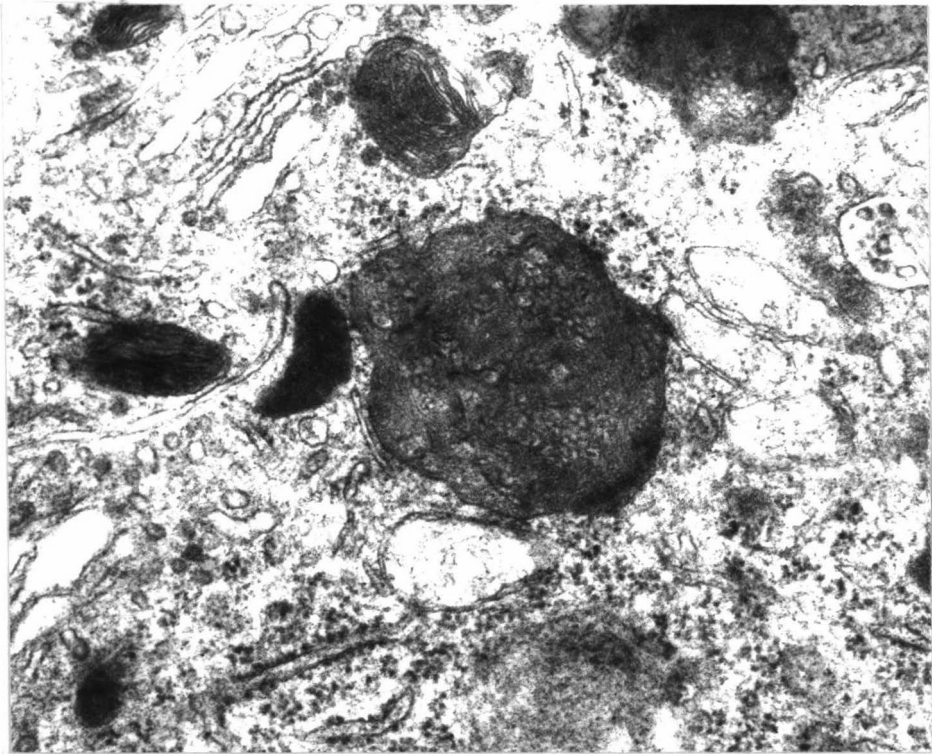
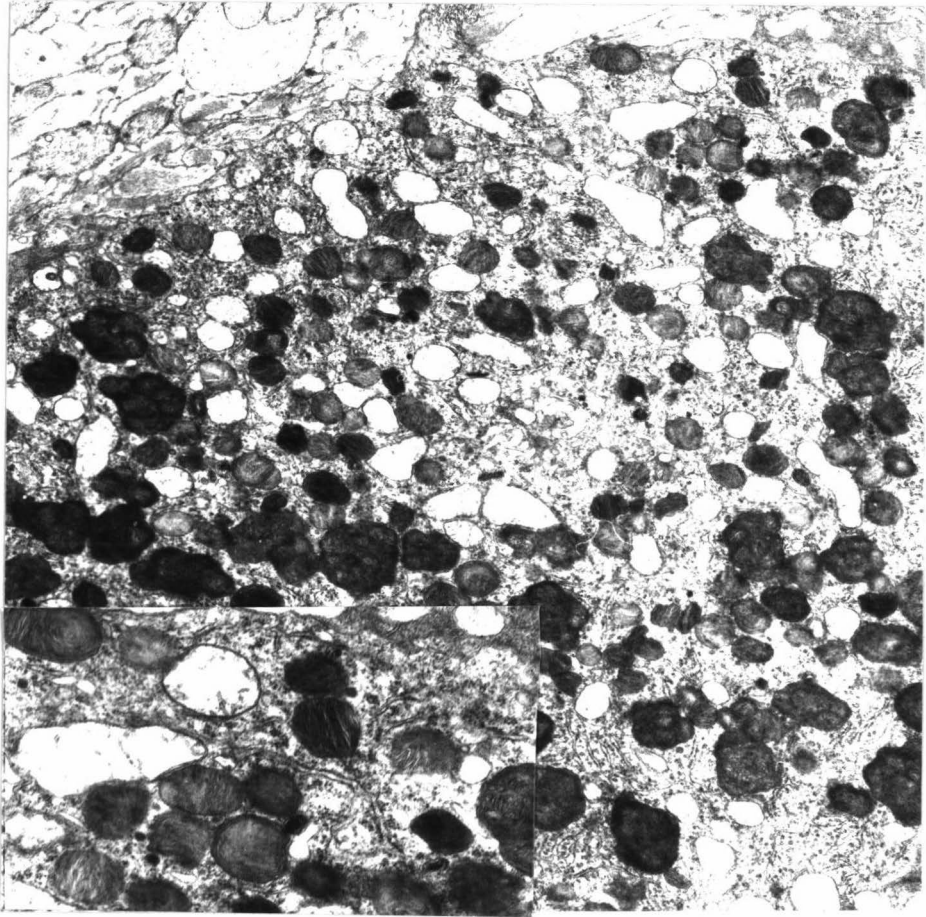


Figure 4.14: A ganglion cell showing large numbers of electron dense ceroid-lipofuscinosis storage bodies. (EM x6300)

The inset shows the membranous storage bodies at a higher power. (EM x23,600)

Figure 4.15: An electron micrograph of a storage body in a ganglion cell which resembles the 'tubular arrays' that occur in the central nervous system. (EM x60,000)



### Discussion

The late stage morphological changes to the ovine retina are similar to those reported in late infantile and juvenile forms of human ceroid lipofuscinosis. However, melanin pigment migration within the retina, noted in the human disease, was not a feature of ovine ceroid-lipofuscinosis. There was good correlation between light and electron microscopic findings. The most significant findings were the gradual and continuous loss of photoreceptor cells and their outer segments and the presence of large amounts of PAS positive, sudanophilic, autofluorescent lipopigment within most cell types in the retina. Degenerate, pyknotic nuclei in the outer nuclear layer were uncommon and the fate of cells lost from the outer nuclear layer is not known as phagocytic cells were not noted in any of the many sections examined. However, these abnormal photoreceptors may have been phagocytosed by the pigment epithelium as normally occurred to spent photoreceptor outer segments.

There were severe morphological alterations to outer segments consisting of short dystrophic membranes shown in Figures 4.9 to 4.12. This study provides evidence that the production of dystrophic outer segments precedes and may precipitate cell loss from the outer nuclear layer. Morphological changes to the outer segments may reflect alterations to a biochemical pathway or pathways within the cell bodies of the photoreceptors leading to interference with the recycling or production of components of the outer segment membranes. These alterations may indicate changes to the fluidity of these highly membranous structures. Differences in the rate of photoreceptor cell body loss observed between rods and cones was supported by a suggestion that rod cell bodies degenerate rapidly

following loss or damage to their outer segments whereas cone cells were more resistant (Marshall, 1980). Loss of the lateral support of adjacent photoreceptor cells could have led to folding and kinking of the remaining outer segments and to the rounding up of inner segments. This kinking and folding of outer segments may explain, at least in part, the apparent shortening of these organelles which was noticed from early in the disease process.

Cell numbers did not appear to vary in the inner nuclear and ganglion cell layers and apart from storage bodies most appeared to be morphologically normal. However, this does not necessarily preclude the existence of severe functional abnormalities. Some cells in the inner nuclear layer showed production of large amounts of coarse membranous material which sometimes involved the Golgi apparatus and the production of large numbers of what were thought to be secondary lysosomes (Fig. 4.7). The significance of these changes is not understood.

The storage material in retinal cells was morphologically similar to that previously described in the central nervous system and other organs of affected sheep (Jolly *et al.*, 1980; 1982). The variable appearance of storage bodies has not been stressed as their contribution to the pathogenesis of retinal disease is probably minimal. Many of the larger pigment accumulations appeared to be aggregations of smaller bodies. Limiting membranes sometimes occurred giving some of the material some features characteristic of secondary lysosomes but this was by no means a universal finding. The small electron dense smudges which were seen at low power were not apparently associated with a limiting membrane (Fig. 4.6).

Examination of them at higher power revealed a granular matrix in which were embedded membranous profiles which resembled the structure of the larger bodies. These were not associated with any particular intracellular organelle and they resembled similar structures reported in the canine (Koppang 1973/74) and ovine models (Jolly et al., 1982). Although it is realised that they may, in fact, be due to the vagaries inherent in the sectioning and interpreting of three dimensional structures, it is possible that they represent early forms of the larger, more complex bodies and that these may become incorporated into secondary lysosomes by the process of autophagy.

The presence of storage bodies per se does not appear to be responsible for the loss of photoreceptor cells. In the dog model there were large numbers of storage bodies present in the outer nuclear layer and significant electroretinographic abnormalities, but blindness and pathology were minimal. Abnormalities reportedly occurred late in the disease process but these have not been fully documented and may be age dependant (Koppang, 1982; Nilsson et al., 1983). In the ovine disease serious morphological abnormalities in outer segment structure appear to contribute significantly to the development of blindness but blindness may also have a central component due to atrophy of the occipital region of the brain.

## CHAPTER 5

## GENERAL DISCUSSION

The initial clinical manifestation of ovine ceroid-lipofuscinosis was loss of vision and as such it most closely resembled the juvenile form of the human diseases. Unlike the human disease, pigment abnormalities in the fundus were not noted and only late in the disease was there slight attenuation of the retinal vasculature.

In the present time course study of the development of retinal pathology, electroretinograms (ERG) were used to plot the decline in visual function with increasing age. This revealed a decline in the 'b' wave amplitudes of rods and cones but with changes to rod responses occurring earlier and being more severe than those of the cone receptors. The 'b' wave is thought to arise in the inner nuclear layer, probably from the Muller cells (Tomita and Yanagida, 1981). Thus changes to it may reflect changes at any level from the outer segments to the Muller cells but the findings reported in the pathology chapter of this thesis suggest that loss of ERG responses may be due to damage to outer segments. Although there was considerable variation in the ERG amplitudes recorded from individual sheep and between sheep there was never the less a rapid decline over a short period of time in the natural progression of the disease. Thus sequential electroretinography could provide a useful method of

monitoring the progression of the disease and may be of value in assessing potential therapeutic strategies.

Pathological studies reported in this thesis confirmed the ERG results and showed a rapid decline in the numbers of photoreceptor cell nuclei present in the outer nuclear layer with increasing age. Initially the majority of the cells lost were rod photoreceptors but later in the disease process cone photoreceptors were also lost. Cell loss was preceded by an apparent shortening of the outer segments. It may be that the accelerated loss of rod photoreceptor cell bodies compared with cones reflects the predominance of this cell type in the sheep retina rather than differing susceptibilities to the disease. There has also been a suggestion that rod photoreceptor cell bodies degenerate and are lost more rapidly than those of cones following damage to their outer segments (Marshall, 1980). There appeared to be no loss of cells from any other layer in the retina. Conspicuously absent throughout the period of study were the phagocytic cells which have been reported in the retina of human cases. The reason for this was not clear but it may reflect the late stage at which human eyes were available for study. Perhaps the abnormal photoreceptors were removed by the highly phagocytic pigment epithelium until very late in the disease process and only after this stage has been reached do phagocytes move into the retina to phagocytose the remaining cellular debris.

Ultrastructurally, the most significant finding was the presence of abnormal dystrophic outer segments of both rod and cone photoreceptors. The reason for this abnormality was not clear but it may reflect the basic biochemical defect in ovine ceroid-lipofuscinosis and as such is worthy of further detailed

investigation. It may be due to primary damage to these highly membranous organelles or it may be due to injury to the cell bodies or to a defect in the normal recycling of biochemical components between the pigment epithelium and the photoreceptors. These changes to the outer segments were accompanied by the presence of abnormal stored lipopigments in the cell bodies.

Storage bodies found in the retina were of similar appearance those described in the central nervous system of the sheep and occurred in most cell types in the retina (Jolly *et al.*, 1980; 1982). In this thesis the morphological diversity of stored lipopigments has not been emphasised because their nosological significance remains unknown. In canine ceroid-lipofuscinosis massive numbers of storage bodies occurred throughout the retina but pathological changes were minimal and functional changes were only reported very late in the disease process (Koppang, 1982; Neville *et al.*, 1980; Nilsson *et al.*, 1983). These findings suggested that the presence of pigment per se was not detrimental to the photoreceptors.

Some of the stored material seemed to be made up of aggregations of smaller bodies whilst others were membrane bound, a feature which was compatible with the structure of secondary lysosomes. In some instances, smudges of electron dense material were found in the cytoplasm of cells and examination of these at very high magnifications revealed the presence of membranous structures within them. While it was appreciated that these could arise due to the vagaries of sectioning three dimensional structures it was tempting to speculate that they represented an early development of storage

material which reached its final form by a process of aggregation and incorporation into secondary lysosome like structures.

The underlying biochemical abnormality(ies) in the ceroid-lipofuscinoses remains an enigma and over the years a variety of pathogenic mechanisms have been postulated. The presence of stored fluorescent lipopigments with staining properties similar to ceroid and lipofuscin gave rise to the name for this group of diseases. These pigments are thought to arise via free radical mediated peroxidation of unsaturated fatty acids so it was not surprising that a similar pathogenesis was proposed for this group of diseases. This theory received some experimental support following the discovery of deficiency of p-paraphenylene diamine mediated peroxidase in the leucocytes of some patients with ceroid-lipofuscinosis (Armstrong et al., 1974). Later studies showed that the apparent deficiency was more likely to be due to abnormal partitioning of the enzyme between the free and bound states (Armstrong, 1982). The presence of ceroid could be due to the autoxidation of abnormal amounts of stored lipid. Other hypotheses for the pathogenesis of these diseases involved anomalies in the metabolism of dolichols and retinoids (Wolfe et al., 1977; 1981).

Results reported in this study suggest that the dystrophic changes to the membranous outer segments may be a primary change of pathogenic significance. In view of this, additional biochemical and ultrastructural studies of this organelle are warranted. Radio isotope labelling studies could be undertaken to investigate the dynamics of photoreceptor production and turnover. Additional ultrastructural studies should focus on this organelle in an attempt

to define the early morphological abnormalities. Methods exist for the isolation of outer segments and biochemical characterization of these may be of benefit. Further electroretinographic investigations could be concentrated on examining the 'c' wave and investigating the central nervous systems contribution to blindness.

Ovine ceroid-lipofuscinosis is an important model of these diseases because of its usefulness in the study of the development of physiological, biochemical and pathological changes using techniques which are unavailable or inappropriate in human medicine.

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