NEUROPATHOLOGY OF OVINE CEROID-LIPOFUSCINOSIS

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Akinori Shimada

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From 

ABSTRACT

The purposes of this study were to describe the neuropathology of ovine ceroid-lipofuscinosis, to compare findings with those of the related entities in humans and other domestic animals, and to provide morphological information that might help elucidate the pathogenesis of these diseases. An established flock of South Hampshire sheep carrying the ceroid-lipofuscinosis gene have made it possible to perform a longitudinal study on the central nervous system of affected sheep of various ages including foetuses.

The most striking gross pathological change of affected sheep was brain atropy. At terminal disease, the brain weights of affected sheep were 55% of those of normal sheep. Atrophy affected mainly the cerebrum.

Sudan black and luxol fast blue positive autofluorescent neuronal pigment granules were detected by lightmicroscopy as early as the mid stage of foetal development, the earliest stage examined. Postnatally there were topographical differences in the quantity of accumulated lipopigments in neurones of various areas. Similarly, there were age related topographical differences in secondary degenerative changes. Neuronal loss was most severe in the parietal lobe cortex showing an initial laminar distribution. This pattern was well demonstrated by a concomitant astrocytosis.

In addition to the complex electron dense cytosomes similar to those reported in the human syndromes, there were less complex cytosomes of smaller size in affected foetal brains. The latter were clearly bounded by a trilaminar membrane and contained whorls or loose stacks of trilaminar membranes resembling those of the limiting membranes. In some electronmicrographs there was a suggestion of continuity between the surrounding membrane and

the internal membranes, but this was not definitely demonstrated. This is provisionally interpreted as being due to an internalization of surrounding limiting membrane rather than a recycling of membrane. Some of these small cytosomes also showed complex multilamellar profiles similar to those of large complex cytosomes. These latter appeared to be formed by coalescence of smaller complex ones. There thus appeared to be a sequence of changes in the development of storage cytosomes.

This study revealed that the ovine disease has not only many neuropathological findings in common with analogous human diseases, but also some pathological features which have not been reported in affected humans or animals. Ovine ceroid-lipofuscinosis is thus a useful animal model for the study of the human ceroid-lipofuscinoses.

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INTRODUCTION

The ceroid-lipofuscinoses, which are also referred to as the neuronal ceroid-lipofuscinoses (NCL), generalized ceroid-lipofuscinoses or Batten's disease are heritable diseases both in humans and in a number of animal species. Affected patients show progressive mental and motor deterioration, loss of vision, seizures and premature death. Pathologically, the common denominator in these diseases is the intracellular accumulation of autofluorescent lipopigments in neurones and a wide variety of extraneural tissues. Only in the nervous system, however, is there secondary degenerative disease leading to brain and retinal atrophy. Despite salient clinical and pathological features of this disease group being described in detail, the biochemical abnormalities are still unknown.

The main purposes of the study reported in this thesis were to describe the neuropathology of ovine ceroid-lipofuscinosis, to compare findings with those of the related entities in humans and other domestic animals, and to provide morphological information that might be useful in elucidating the pathogenesis of the disease. For these purposes, chronological and topographical studies were performed on the central nervous system.

CHAPTER I

REVIEW - THE NEURONAL CEROID-LIPOFUSCINOSES

I. HISTORICAL ASPECTS

The first cases of neuronal ceroid-lipofuscinosis (NCL) were probably reported by Stengel (1826) who described four affected siblings in a small mining community in Norway. They showed progressive visual failure, epileptic seizures, speech difficulties, sensory-motor regression, and profound mental dullness by the age of 15 years. Two patients died at the ages of 20 and 21 years, the other two being alive and aged 8 and 16 years at the time of reporting. Although no histological studies could then be performed, the description of clinical findings was so succinct that a retrospective diagnosis of the juvenile form of ceroid-lipofuscinosis is probably justified.

In 1903, Batten reported two sisters whose initial presenting signs were failing vision at about the age of 6 years followed by dementia. He described cerebral degeneration with symmetrical changes in the macula, slight pallor of the optic discs, and retinal pigmentary changes with irregular poorly defined reddish-black spots in the macula. In the next year Mayou (1904) reported three siblings with cerebral degeneration and similar macular changes. Spielmeyer (1905), Vogt (1905), Jansky (1908) and Bielschowsky (1913) described similar disorders. These reports indicate that the authors grouped together what are now regarded as different types of the syndrome. Although at first Batten considered that the pathological findings of his and other cases were different from those of "amaurotic family idiocy" (Sachs, 1896) which was named as "Tay-Sachs disease" by Higier (1901), he later changed

his opinion. He listed three types of amaurotic idiocy with different ages of onset, infantile (Tay-Sachs disease), late infantile and juvenile amaurotic idiocy as a group of diseases characterized by maculocerebral degeneration (Batten & Mayou, 1915).

In 1931, the Swedish psychiatrist and geneticist, Torben Sjögren, presented 115 cases that had been subjected to an extensive clinical and genetic investigation. He concluded that the disease, which he called the Spielmeyer-Sjögren type or "juvenile amaurotic idiocy", was genetically different from Tay-Sachs disease (infantile amaurotic idiocy). Today this latter disease is identified biochemically as the infantile form of G_{M2}-gangliosidosis. Kufs (1925) also described an adult form of amaurotic idiocy. These well-documented reports had little impact upon views of neurologists, geneticists and neuropathologists, and the common concept during the 1950's and 1960's was still that the various types of amaurotic idiocies, of which Tay-Sachs disease was the prototype, were age-dependent variants of one and the same basic pathological process.

Zeman & Alpert (1963) made a resolute effort to describe the pigmentary nature of the neuronal deposits in various types of familial amaurotic idiocy. Terry & Korey (1960), and Terry & Weiss (1963) specified ultrastructural and biochemical findings for Tay-Sachs disease, and these results contributed to the separation of the disease from the other amaurotic idiocies by Zeman & Donahue (1963). At that time it was suggested that the so-called late infantile amaurotic idiocy of Jansky-Bielschowsky, the juvenile amaurotic idiocy of Spielmeyer-Vogt, and the adult form of amaurotic idiocy of Kufs, were distinct from Tay-Sachs disease and other forms of sphingolipidosis (Donahue et al., 1967). Subsequently Santavuori et al., (1973)

and Haltia et al., (1973a, 1973b) showed that an infantile form of neuronal ceroid-lipofuscinosis also existed, which had an onset of clinical signs during the first or second year of life with early development of blindness and a rapid course.

Infantile (Haltia-Santavuori), late infantile (Bielschowsky-Jansky), juvenile (Batten-Mayou, Spielmeyer-Vogt, Spielmeyer-Sjögren) and adult (Kufs) form are now generally classified under the general heading of "neuronal ceroid-lipofuscinosis", a term first used by Zeman & Dyken in 1969. Eponymic names are still frequently used and Batten's disease is sometimes used loosely for the whole group. The term "neuronal ceroid-lipofuscinosis" is mainly used in American literature but is not completely satisfactory as pigment is found also in other tissues in the body. The alternate name "generalized ceroid-lipofuscinosis" has been suggested by Joosten et al. (1973). Subgroups called "early juvenile" (Lake & Cavanagh, 1978; Libert et al., 1982) and "protracted juvenile form" (Goebel et al., 1976; Libert et al., 1982) were later reported. There are also other atypical cases (Greenwood & Nelson, 1978; Jervis & Pullarkat, 1978; Goldman et al., 1979; Proops et al., 1981; Santavuori et al., 1982; Ikeda et al., 1984; Goebel, 1985). The main types are summarized in Table 1.I.

It must be emphasized that a rational nomenclature does not become possible until the specific genetically determined biochemical defects are understood.

II. CLINICAL FEATURES

The common clinical features of the neuronal ceroid-lipofuscinoses are visual loss, progressive mental and motor deterioration, seizures and premature death (Zeman & Siakotos,

1973; Zeman, 1976). Lymphocytic vacuolation and hypergranulation of neutrophils may be found with a high degree of frequency in some forms (Zeman, 1976). The presenting signs differ from one type to the other (Table 1.I). However, the validity of separating these syndromes clinically must be substantiated by further pathological and biochemical characterization of the various forms of this disease.

Infantile form of the disease

The patients developed normally for up to 8-16 months, reaching their milestones at the expected times, and then presented with rapidly progressive psychomotor retardation. This was accompanied by hypotonia and ataxia. Myoclonic jerking and visual loss followed but fits were not a prominent feature. All patients showed microencephally and reached a "burnt out" state by 3 years of age. The electro-encephalogram (EEG) became isoelectric by this time. There was no lymphocytic vacuolation but azurophilic hypergranulation of neutrophils was occasionally found. Death occurred at between 3 and 10 years. (Santavuori et al., 1973; Haltia et al., 1973a, 1973b)

Late infantile form of the disease

This was a rapidly progressive disorder with early onset between 18 months and 4 years. There was insidious impairment of vision, spastic tetraplegia and bulbar paralysis leading to death at 4-10 years of age. There were massive convulsions which resulted in death after a relatively short duration of the illness, in some instances as little as 9 months. Azurophilic hypergranulation of neutrophils was frequently observed. (Zeman & Siakotos, 1973; Zeman, 1976).

Juvenile form of the disease

This form of ceroid-lipofuscinosis usually began with visual disturbances between the ages of 4 and 9 years which were associated with a pigmentary retinopathy. Minor psychological

TABLE 1.I

CLINICAL CLASSIFICATION OF THE NEURONAL CEROID-LIPOFUSCINOSES

Type (Eponym)	Age of onset	Symptoms	Outcome	White blood cell	Key reference
Infantile (Haltia-Santavuori)	8-16 month	Myoclonus, visual failure, Psychomotor retardation, ataxia	Rapidly fatal	Normal	Santavuori et al.,(1973); Haltia et al., (1973a, 1973b)
Late infantile (Bielschowsky-Jansky)	18 month-4 year	Regression of skills, speech difficulty, convulsions, myoclonus, visual failure, ataxia	Relatively short survival	Hypergranulation in neutrophils	Zeman & Siakotos,(1973), Zeman,(1976)
Juvenile (Battern-Mayou Spielmeyer-Vogt; Spielmeyer-Sjögren)	4-9 year	Blindness, convulsions, dementia, behavioural changes	Variable; fatal within months or several years	Lymphocytic hypergranulation in neutrophils	Boehme et al., (1971);Zeman & Siakotos,(1973) Zeman,(1976); Lake, (1977)
Adult (Kufs)	15 year	Dementia, behavioural changes, convulsions	Slow progression	Hypergranulation in neutrophils	Boehme et al., (1971); Zeman, (1976)

deviations and alterations in motor behavior accompanied the onset of these features. There followed gait disturbances, slurred speech and anxiety. Finally, the patients became completely withdrawn and bedridden with contractures of their limbs. Spastic tetraplegia led to death in the late teens or early twenties but older patients are known (Boehme et al., 1971; Zeman, 1976). Cytoplasmic vacuoles (Bagh & Hortling, 1948) as well as cytoplasmic inclusions (Nasu et al. 1969) in peripheral blood lymphocytes have been regarded as morphologic markers of the juvenile type of the neuronal ceroid-lipofuscinosis (Zeman & Dyken, 1969; Kristensen & Low, 1983). There are differing opinions as to whether significant lymphocytic vacuolation occurs in heterozygotes of this type of the disease (Rayner & Book, 1958; Witzleben, 1972; Bürrig et al., 1982). Azurophilic hypergranulation of neutrophils has also been reported frequently in the juvenile form of the disease (Zeman & Strouth, 1967; Zeman & Siakotos, 1973). It was also found to be a "reliable, conspicuous, and consistent marker" for the defective gene in heterozygotes (Zeman & Strouth, 1967).

Adult form of the disease

The adult form of neuronal ceroid-lipofuscinosis differed from the other forms in that visual disturbances were rare (Dom et al., 1979). This was a relatively milder form of the disease which usually began during adulthood. However, behavioural disturbances with progressive dementia, ataxia and grand mal fits might occur at any time from childhood to adult life. Extrapyramidal signs and cerebellar symptoms followed. Slowness of speech leading to dysarthria was a feature. The disorder produced less severe mental disturbances than other types of the disease. No lymphocytic vacuolation has been reported, but azurophilic hypergranulation of neutrophils was occasionally found. (Boehme et al., 1971; Zeman, 1976).

The four forms of ceroid-lipofuscinosis described above, have been adopted as a general classification (Zeman, 1976). However there were other variants which did not exactly fit the previous four types. These included an early-juvenile disease with the early presentation similar to late-infantile form of the disease but whose course and pathological changes were similar to those of the juvenile form (Lake & Cavanagh, 1978; Libert et al., 1982). Similarly there was a protracted juvenile form of the disease in which clinical signs were similar to those of the juvenile form but occurred later and continued over a longer time (Goebel et al., 1976; Libert et al., 1982). Occasional atypical cases have been reported whose symptoms and clinical course varied from the above (Greenwood & Nelson, 1978; Jervis & Pullarkat, 1978; Goldman et al., 1979; Proops et al., 1981; Santavuori et al., 1982; Ikeda et al., 1984; Goebel, 1985).

When more than one patient was affected in a family they tended to exhibit onset of clinical signs at a similar age (homochronism) and the disease ran a similar course (homotypism) (Zeman, 1976). This tends to confirm the fact that the syndromes described do reflect different genetic forms of the disease.

III. INHERITANCE AND PREVALENCE

The neuronal ceroid-lipofuscinoses are uncommon diseases but they have been reported in most common races and ethnic groups, and in both sexes (Zeman et al., 1970). An autosomal recessive mode of inheritance is evident for most of the forms. An exception is a possible dominantly inherited form of Kufs type which occurred in five generations of a family (Boehme et al., 1971; Leonberg et al., 1982).

Santavuori et al. (1974) stated that the prevalence of the infantile form in Finland was 7.85 per 100,000. In Sweden, Sjögren (1931) estimated that the frequency of the juvenile form of the disease was 1 in 25,000 and Rayner (1962) reported the prevalence of the same form of the disease at 1 in 50,000. Reske-nielsen et al. (1981) estimated the incidence of the juvenile form at 1-3 in 100,000 per year and reported that by November 1980, 21 patients affected with juvenile form were registered in Denmark with a population of 5 million. About "one dozen" new cases of the neuronal ceroid-lipofuscinoses are reported per year in the United Kingdom and of these the juvenile form accounted for one quarter (Henry & Stevens, 1982).

The prevalence of the late infantile and adult forms is less well known.

IV. PATHOLOGICAL CHANGES

The progressive accumulation of autofluorescent lipopigments in neuronal perikarya and a wide variety of other cells is the common denominator in the neuronal ceroid-lipofuscinoses. However only in the nervous system is there secondary degenerative disease leading to brain and retinal atrophy (Zeman, 1970, 1976).

Gross pathology

The striking gross abnormality in all forms of the disease was cerebral atrophy (Zeman et al., 1970). The white matter was firm. In the infantile form of the disease, the dura as well as the skull was thickened and the latter was up to 1.5 cm thick (Haltia et al., 1973a; Hagberg et al., 1974; Santavuori et al.,

1974). In the late-infantile form of the disease, atrophy of the cerebellum was pronounced (Smith et al., 1981). Using cranial computed tomography (CCT), Lagenstein et al. (1981) reported symmetrical enlargement of subarachnoidal spaces and of the lateral, third and fourth ventricles.

Light microscopy

There were differences in the extent of neuronal and extraneuronal involvement among the types of ceroid-lipofuscinosis. The main lesions consisted of pigment accumulation, neuronal death and glial reaction in the nervous system.

The brain of the infantile type showed progressive loss of nerve cell bodies throughout the cortex and of myelin and axons in the white matter (Zeman, 1976; Haltia et al., 1973a, 1973b). Up to 2.5 years neuronal storage of pigments was observed and there was some degree of neuronal loss. There was an astrocytosis and presence of the macrophages. From the age of 2.5 years the neurones became greatly depleted in number, astrocytes contained stored material and macrophages became more numerous. Loss of myelin was evident. By 4 years cortical neurones and associated axons and myelin sheaths had mainly disappeared. The cerebrum was largely replaced by fibrillary astrocytes which contained lipopigments (Santavuori et al., 1974). Neurones in the brain stem, basal ganglia and spinal cord were distended by large amounts of storage pigments.

In the late infantile form of the disease de Baecque et al. (1976) described a reduction in the numbers of the Purkinje cells, a striking loss of granular cells and a prominent Bergmann cell gliosis in the cerebellum. The white matter of the brain showed mild demyelination and proliferation of astrocytes (Dolman et al., 1972; de Baecque et al., 1976). A

status spongiosus of the forebrain cortex has been reported as a peculiar cortical lesion (Bielschowsky, 1913; Seitelberger, 1962; Dolman et al., 1972; Zeman, 1976), and interpreted as a seizure-induced anoxic-ischemic phenomena by Zeman (1976).

Some reports described only a scant numerical reduction of cortical neurones and an apparently normal cortical lamination in the juvenile form of the disease (Suzuki et al., 1968; Williams et al., 1977). However this is probably erroneous as using preparations stained with aldehydefucshin, Braak & Goebel (1978) demonstrated severe structural alterations of the cortex in the case of juvenile type of the disease. This affected a single cell type, in particular the small pigment-laden stellate cells of the isocortex, resulting in their complete loss of layer II. They postulated that the selective involvement of these local circuit neurones might be due to their small size and the early onset of lipopigment accumulation and be causally related to the functional impairment of the brain. Furthermore they demonstrated that the pyramidal cells of the upper parts of the pyramidal layer (IIIab) tended to develop giant fusiform swellings of their axonal initial segments which were filled with lipopigment granules. The ganglionic layer (V) was also severely depleted of pyramidal cells and considerably attenuated with a characteristic "pepper and salt" appearance (Braak et al., 1979). They indicated that those cortical neurones probably linked by interlaminar connections showed the most marked cytopathologic features. The appearance of lipofuscin-laden appendages emerging from IIIab-pyramidal cells is also a characteristic feature of the aging human brain (Braak, 1979).

Pigment architectural analysis of the brains of one case of protracted juvenile form and two cases of adult form of ceroid-lipofuscinosis also revealed loss of pigment-laden stellate

cells in layer II, axonal enlargement of layer IIIab-pyramidal cells, and considerable cell loss in layer Va (Goebel et al., 1982b). It was suggested that this pattern of cortical pathology was specific for the neuronal ceroid-lipofuscinoses (Goebel et al., 1982a).

The storage pigments were acid-fast, PAS-positive and stained with Sudan black B. They also stained positively with luxol fast blue except for those in the infantile form of the disease (Lake, 1984). They were autofluorescent in ultraviolet light.

The retinas in terminal disease showed severe atrophy with loss of the photoreceptor cells. The ganglion cells were also lost in the infantile form, but were relatively well preserved in the other forms but with lipopigment accumulation in their cytoplasm. The optic nerve was atrophic in the infantile and late infantile forms of the disease (Zeman, 1976).

In the infantile form of neuronal ceroid-lipofuscinosis, abnormal accumulations of lipopigments have also been reported in epithelial cells of the thyroid, pancreas, kidney and lung; testis; skeletal, cardiac muscle and smooth muscle of the intestinal wall; macrophages in spleen, liver, lymph nodes, bone marrow and lamina propria of intestinal mucosa; adventitial mesenchymal of small vessels and neurones of the gastrointestinal tract (Haitia et al., 1973b; Hagberg et al., 1974; Siegismund et al., 1982).

In the late infantile form of the disease, intracytoplasmic accumulations of coarse and fine pigment granules were found in cells of heart, aorta, lung, pituitary, thyroid and adrenal glands, bone marrow, lymph nodes, kidney, urinary bladder, striated muscle, sweat gland epithelium and liver (Dolman & Chang, 1972; de Baecque et al., 1976).

Lipopigments were found in cells of the following tissues or organs of the patients with juvenile form of ceroid-lipofuscinosis: dorsal root ganglion cells, neurones of the autonomic nervous system of the intestinal wall, myocardial muscle, epithelium of the distal part of collecting tubules in the kidneys, large "reticulo-endothelial" cells in spleen, thymus, lymph node and bone marrow, parenchymal and Kupffer's cells in the liver with a predominantly centrilobular pattern, the anterior lobe of the pituitary, pancreas, thyroids, gonads and sural nerve (Kristensson et al., 1965; Nakano et al., 1978). Small numbers of intensely acidphosphatase-positive histiocytes were found between the smooth muscle cells in rectal biopsies. Miley et al. (1978) described the presence of sea blue histiocytes in the bone marrow of a 6 years old boy with juvenile ceroid-lipofuscinosis. It was, however, found to be a non-specific change (Gadoth, 1982). Recently Reske-nielsen et al. (1981) reported the cardiac involvement in 13 patients affected with juvenile ceroid-lipofuscinosis, in which they demonstrated that all compartments of the heart including the conduction system were involved. There was a discrepancy between the relatively minor functional disturbances observed and the severe morphological changes in the heart.

Electron microscopy

At one time, the neuronal ceroid-lipofuscinoses were classified with the gangliosidoses. However electron microscopic studies by Terry & Korey (1960), Terry & Weiss (1963) and Zeman & Donahue (1963) led to the separate classification of these diseases (Zeman & Donahue, 1963).

Koenig et al. (1964), Gonatas et al. (1968) and Siakotos et al. (1972) suggested their probable lysosomal association by demonstrating acid phosphatase activity in the accumulated lipopigments of the neuronal ceroid-lipofuscinoses.

The lipopigments were bounded by a unit membrane (Donahue et al., 1967). They were irregular in shape and varied in size. Although the fine structures of the lipopigments have been reported differently among cases affected with ceroid-lipofuscinosis, the internal structure of the lipopigment cytosome was generally composed of a granular matrix and a variety of membranous profiles. The latter consisted of alternating dense and pale bands. The arrangement and shape of these lamellae was irregular, presenting round, oval or tubular structures (Zeman & Siakotos, 1973). The cytosomes with predominantly membranous profiles were described as multiloculated lipid bodies (Zeman & Donahue, 1963), multilamellar cytosomes (Gonatas et al., 1968), curvilinear bodies (Duffy et al., 1968) and inclusions with fingerprint pattern (Suzuki et al., 1968). The term zebra bodies (Elfenbein & Cantor, 1969; Zeman et al., 1970; Herman et al., 1971), membranous cytoplasmic bodies (Sluga & Majdetzki, 1967), and crystalloid inclusions (Donahue et al., 1966) have also been used to describe these lipopigments.

Characteristic profiles of the lipopigment granules described as curvilinear bodies, fingerprint pattern and granular internal structure, have been thought to be specific to some types of the neuronal ceroid-lipofuscinosis (Gonatas et al., 1968; Carpenter et al.,1977). There are, however, many reports on the fine structure of the lipopigment, which show inconsistency between the ultrastructural patterns and the type of neuronal ceroid-lipofuscinosis (Zeman et al., 1970; Herman et al., 1971; Dyken & Trefz, 1979; Goebel et al., 1979). Zeman et al. (1970) minimized the significance of differences in the fine structure of lipopigment bodies. Goebel et al. (1979) concluded that the ultrastructural differences were more of a quantitative than of a qualitative nature, and a function of

age-dependent and local tissue and cellular biochemical factors, rather than a disease-specific phenomenon.

Due to the presence of residual bodies showing pathognomonic structures with obvious diagnostic value in the neuronal ceroid-lipofuscinoses, biopsy has been applied as a diagnostic tool (Table 1.II) in skin (Carpenter et al., 1972; Dolman et al., 1975; Ceuterick et al., 1976; Farrell & Sumi, 1977; Sipe & Obrien, 1979; Ishii et al., 1981; Henry & Stevens, 1982; Finkel et al., 1983), skeletal muscle (Goebel et al., 1975; Dom et al., 1979; Vercruyssen et al., 1982), sural nerve (Joosten et al., 1973), autonomic ganglia and macrophages in rectal mucosa (Lake, 1977; Lake & Cavanagh, 1978; Siegismund et al., 1982; Rapola et al., 1984), appendix (Van Haelst & Gabreels, 1972; Rapola & Haltia, 1973) and urinary sediment (de Baecque, 1975; Armstrong et al., 1977; Dolman et al., 1980), dental pulp (Witkop et al., 1984) and amniotic fluid cells (MacLeod et al., 1985).

V. THE NEURONAL CEROID-LIPOFUSCINOSES IN DOMESTIC ANIMALS

There are several species of animal which have been reported with syndromes analogous to ceroid-lipofuscinosis. They are Beefmaster cattle (Read & Bridges, 1969), English Setter dog (Koppang, 1970, 1973/74; Armstrong et al., 1982), Chihuahua dog (Rac & Giesecke, 1975; Jolly & Hartley, 1977), Dachshund dog (Cummings & de Lahunta, 1977; Vandevelde & Fatzer, 1980), Saluki dog (Appleby & Longstaffe, 1982), Cocker Spaniel dog (Wilkie & Hudson, 1982), Dalmatian dog (Goebel & Dahme, 1985), and Blue Heeler dog (Cho et al., 1986; Wood et al., 1987), South-Hampshire sheep (Jolly et al., 1980) and Siamese cat (Green & Little, 1972). The clinical signs and the ultrastructural findings of the storage material varied in these animals.

TABLE 1.II

TISSUES VALUABLE IN MORPHOLOGICAL DIAGNOSIS IN THE NEURONAL CEROID-LIPOFUSCINOSES

Tissue	Reference
Skin	Carpenter et al.,1972; Dolman et al.,1975; Ceuterick et al.,1976; Farrell & Sumi,1977; Sipe & Obrien,1979; Ishii et al.,1981; Henry & Stevens,1982; Finkel et al.,1983
Skeletal muscle	Goebel et al.,1975; Dom et al.,1979; Vercruyssen et al.,1982
Sural nerve	Joosten et al.,1973
Rectal mucosa	Lake,1977; Lake & Cavanagh,1978; Sigismund et al.,1982; Rapola et al.,1984
Appendix	Van Haelst & Gabreels,1972; Rapola & Haltia, 1973
Urinary sediment	de Baecque,1975; Armstrong et al.,1977; Dolman et al.,1980
Amniotic fluid cell	MacLeod et al.,1985
Dental pulp	Witkop et al.,1984

Ceroid-lipofuscinosis in English Setter dogs was established as a model for the juvenile type of human ceroid-lipofuscinosis through detailed clinical, pathological, and biochemical investigations (Koppang, 1973/74). Affected animals were clinically normal up to the age of 12 to 14 months. They usually showed the first sign of disease around the age of 14 months to 18 months, when reduced vision and mental dullness became obvious. From 17 to 24 months, the animals became ataxic, their extremities stiffened and they grew progressively blind. Convulsions and contractions set in at the latter stage of the disease and death usually occurred before 25 months of age.

Homozygous-affected dogs crossed with normal dogs produced no affected animals whereas heterozygous matings produced 25% affected puppies and homozygous-homozygous, 100%. Therefore an autosomal recessive mode of inheritance of the disease was clear (Koppang, 1973/74).

In the later stage, especially after 20 months of age, the dura was thickened. The brain was firm and atrophic weighing approximately 60-70% of normal age-matched unaffected littermates. Dilated ventricles and discoloured yellow-brown cortex were also noted.

From birth to 2 months of age, autofluorescent pigments were difficult to detect with light microscopy (Koppang, 1973/74). PAS and Sudan black B positive pigments could be found in about 30% of neurones of all regions of the central nervous system by the age of 2-3 months, and in all neurones at the age of 6 months. The amount and fluorescence of the lipopigments increased gradually in the neuraxial nerve cells and peripheral neurones. By the age of 12 months, pigments occupied the "whole cytoplasm" in a large percentage of neurones.

Such cells might reveal nuclear pyknosis, rounding of the soma and loss of Nissl substance (Koppang, 1973/74). Loss of neurones was noted to commence about the age of 12-14 months, and by terminal disease had "denuded man' grisea", particularly of the cerebellar cortex. Loss of neuronal perikarya was inevitably associated with the loss of central and peripheral nerve processes. In the latter, demyelination and axonal degeneration could be observed. Recently, Braak et al. (1984) demonstrated pigment-filled enlargements of the initial axon segments of cortical layer IIIab pyramidal cells.

Retinal nerve cells contained large quantities of autofluorescent pigments at the time of death with minor degenerative changes and occasional cell death thereof. In contrast to the human disease, the photoreceptor cells were preserved. Cells of visceral organs and other extraneuronal tissues also showed a gradual increase in the autofluorescent pigment.

Electron microscopic observations revealed the presence of "cytoplasmic condensation" in neurones of central nervous tissue at 2 days of age. The condensations or focal densities with a granular or almost homogeneous pattern measured less than 1 µm in diameter and were randomly distributed throughout the cytoplasm. They were considered to be a "pigment" or precursors of the autofluorescent pigments to which they were transformed by autophagy and formation of residual bodies.

Typical membrane-bound ceroid bodies, 0.1 to 1.0 µm in diameter, were frequently found shortly after birth. Therefore it was suggested that pigment formation began in utero. The number and size of accumulating pigment granules increased as a function of time to the age of 12 months. There was a

concomitant numerical loss of subcellular organelles such as mitochondria, endoplasmic reticulum and ribosomes. By the age of 12 to 25 months, neuronal degeneration and necrosis and accumulation of pigment in astrocytes, oligodendrocytes and endothelial cells were observed in the gray matter. Pigment laden astrocytes and oligodendrocytes were also found throughout the white matter.

The ultrastructural pattern of the pigment bodies was variable between different animals, different topographical areas of the brain, and different cell types. The pigment bodies commonly contained five-layered membranous profiles separated by a granular osmophilic matrix which were called curvilinear bodies.

Electronmicroscopically, photoreceptor outer segments of the retina appeared damaged and pigment laden phagocytic cells were often seen at the photoreceptor-retinal pigment epithelium interface. Characteristic pigment bodies of the same architecture as described above were observed in extraneuronal tissue, but were not apparently associated with cellular degeneration. Lymphocytes of affected dogs contained the small clear vacuoles in which membrane bound inclusions were present (Goebel et al., 1981).

Electrophysiologic studies have been performed extensively on affected dogs. Reductions in a-wave and b-wave amplitudes were recorded in full-field electroretinograms (ERGs) in three 20 to 22 months old affected dogs (Berson & Watson, 1980). Nilsson et al. (1983) reported an abolished c-wave in the seriously affected dogs. The electroencephalograph (EEG) showed high-voltage slow waves with spikes by 6 months of age, and by terminal disease electrical activity had ceased (Armstrong et al., 1982). Recently Armstrong et al. (1986) also reported

abnormalities such as sinus arrhythmia, A-V dissociation or tachycardia in electrocardiograms recorded in a longitudinal study on six affected dogs.

Two cases of suspected ceroid-lipofuscinosis in two unrelated Chihuahua dogs, a 24 months old male and a 22 months old female, were reported (Rac & Giesecke, 1975). Clinically the affected dogs showed progressive blindness, neurological disturbances and temperament changes at the age of 2 years. Microscopically, eosinophilic, weakly acid fast, PAS and Sudan black B positive granular material in the cytoplasm of the majority of neurones, glial cells, retinal and enteric ganglia, and spleen were observed. Necrosis and loss of neurones in the layer of Purkinje cells and scattered axonal fragmentation in the white matter were also found. Similar cases in Chihuahua dogs have been seen in New Zealand and Australia (Jolly & Hartley, 1977).

Cummings & de Lahunta (1977) reported on the occurrence of an adult case of canine neuronal ceroid-lipofuscinosis in a 4.5 years old Dachshund and suggested it as a possible model for human Kufs type of ceroid-lipofuscinosis. Neurologic signs referable to the cerebellum appeared later in life and progressed slowly. Grossly there was generalized cerebellar atrophy, moderate enlargement of the lateral and fourth ventricles, and a symmetrical distinct yellow discolouration of the cerebellar nuclei. Varying amounts of PAS and Sudan black B positive, autofluorescent granular cytoplasmic material were recognized by light microscopy in the neurones and macrophages throughout the neuroaxis. Widespread loss of Purkinje cells and a status spongiosus in the brain stem and molecular layer of the cerebellum were observed. Electronmicroscopically the intraneuronal membrane-bound cytosomes ranged from 0.4 to 2.2um in diameter and were composed of pleomorphic lipid bodies,

zebra bodies, compound bodies, typical lipofuscin granules, lysosome-like bodies, and membranous cytoplasmic bodies. Vandevelde & Fatzer (1980) also documented the occurrence of two adult Dachshund dogs affected with ceroid-lipofuscinosis. There was considerable similarities in age and morphologic features between these and Dachshund dogs reported by Cummings & de Lahunta (1977).

Two 2-year-old Saluki dogs from related litters exhibited changes similar to the human juvenile type of ceroid-lipofuscinosis (Appleby et al., 1982). Clinically the affected dogs developed nervous signs from 1 year of age and showed inco-ordination, swimming movements and inability to stand. No gross abnormalities were found. Microscopically autofluorescent, luxol fast blue, PAS and Sudan black B positive pigment was demonstrated in the cytoplasm of large neurones of the brain, retina and enteric ganglia. Electron micrography revealed membrane-bound masses of laminar inclusions in patterns of whorls or bundles in the cytoplasm of neurones.

Wilkie & Hudson (1982) described an 18-month-old Cocker Spaniel dog with a progressive central nervous system disorder. There were granular pigments in many neurones as well as in extraneuronal tissues. These granular pigments were PAS and luxol fast blue positive, and autofluorescent under ultraviolet light. Electronmicroscopically they were membrane-bounded and contained laminated structures resembling condensed membrane fragments, amorphous materials, and dense granules.

Details of retinal ultrastructure of two Dalmatian dogs, 1.5 and 7 years old, affected with ceroid-lipofuscinosis were described by Goebel & Dahme (1985). Clinically they showed marked ataxia, blindness, and abnormal behaviour. There was an

ubiquitous accumulation of lipopigments in a variety of cell types of the retina. Their fine structures were described as curvilinear, fingerprint, and rather complex membranous profiles. Photoreceptors and remaining retinal cells were generally preserved.

Cho et al. (1986) reported on the occurrence of a neuronal ceroidosis (ceroid-lipofuscinosis) in an 18-month-old Blue Heeler dog with a progressive gait and behaviour abnormality, depression, paresthesia and deterioration of vision. Grossly the brain was slightly atrophic. Histologically there were autofluorescent, PAS, luxol fast blue, and oil red O positive intracytoplasmic granules in the neurones of the brain. A moderate diffuse reduction in the number of cerebellar and cerebrocortical neurones was observed. Electron micrographs revealed the diversity of the neuronal cytosomes. They were membrane-bound, and were composed of convoluted and stacked membranous profiles and a granular matrix. Occurrence of ceroid-lipofuscinosis in two Australian Blue Heeler dogs, 25 and 26 months old, a male and a female respectively, was also reported by Wool et al. (1987). These two dogs were littermates. Their clinical and pathological features resembled those of the juvenile form of human ceroid-lipofuscinosis.

Green & Little (1974) described neuronal ceroid-lipofuscin storage in two mature Siamese cats, one of which showed convulsions and mania, while the other appeared irritable and had hindleg weakness. Microscopically, luxol fast blue, PAS and Sudan black B positive cytoplasmic inclusions were detected in the neurones. There was a mild diffuse microgliosis in the medulla and cerebral cortex, and some vacuolation of the white matter. The ultrastructure of these inclusions was similar to the curvilinear bodies described in human ceroid-lipofuscinosis. No abnormal quantitative or qualitative changes in brain lipids have been demonstrated by biochemical analyses.

Spontaneous neuronal lipodystrophy involving visceral tissue was reported in an 18-month-old bull of an inbred strain of Beefmaster cattle (Read & Bridges, 1969). Clinically the animal was blind and showed intermittently circling and convulsions. Microscopically all neurones of the central nervous system as well as those of the ganglion cell layer of the retina and of the peripheral plexuses contained intracytoplasmic coarse granules. They were stained with luxol fast blue, oil red O and Sudan black B but were negative with PAS and acid-fast stains. Ultrastructurally inclusions were similar to the curvilinear bodies and multilamellar cytosomes described in ceroid-lipofuscinosis in human. This disease is thus consistent with ceroid-lipofuscinosis.

Jolly & West (1976) reported the presence of a neuronal ceroid-lipofuscinosis in South Hampshire sheep. On the basis of the findings on six affected sheep, it was proposed that ovine ceroid-lipofuscinosis was a useful model for juvenile type of human Batten's disease (Jolly et al., 1980). Strong evidence of an autosomal recessive mode of inheritance was provided by a series of sire/daughter matings (Jolly et al., 1980). The affected sheep were characterized clinically by blindness, behavioural abnormalities, and motor dysfunction.

Grossly the brains showed atrophy with thinning of the gyri, slight enlargement of lateral ventricles and thinning of the septum pellucidum and corpus callosum (Jolly et al., 1980). Histologically intracellular accumulation of autofluorescent, Sudan black B and PAS positive, and slightly acid-fast lipopigments were recognized in neurones and a wide variety of other cells within the body. There was loss of neurones and an astrocytosis in the cerebral cortex. Occasional formation of spiny meganeurites resembling those reported in both human and

canine neuronal ceroid-lipofuscinosis was observed in the neurones of the cerebral cortex (Walkley S.U., pers. comm.).

In the retina most cells showed the accumulation of a fluorescent lipopigment but this was particularly so in ganglion cells (Graydon & Jolly, 1984). Dystrophy of photoreceptor outer segments was observed which preceded necrosis of the photoreceptor cells themselves. A severe and progressive reduction in b-wave amplitudes in the electroretinogram (ERG) accompanied these changes. More recently it was revealed that a diminished c-wave preceded a progressive loss of a- and b-waves during the course of the disease (Samuelson et al., 1985; Mayhew et al., 1985).

Ultrastructurally the lipopigment was granular in appearance with a wide variety of lamellar profiles similar to those reported in the human and canine diseases.

CHAPTER II

GROSS PATHOLOGY

I. INTRODUCTION

In humans, the striking gross abnormality found in patients affected with neuronal ceroid-lipofuscinosis is cerebral atrophy (Zeman et al., 1970). Brain atrophy has also been reported in animals with analogous disease, such as English Setter dog (Koppang, 1973/1974), Dachshund (Cummings & de Lahunta, 1977), Blue Heeler dog (Cho et al., 1986; Wood et al., 1987) and South Hampshire sheep (Jolly et al., 1980; Mayhew et al., 1985).

This chapter describes the pattern and degree of atrophy in affected sheep relative to age.

II. MATERIALS AND METHODS

Animals

The experimental sheep used in this study (Table 2.I experimental cases 4-19) were from an inbred flock of South Hampshire sheep maintained by the mating of homozygously affected 8-9 month males with obligate heterozygous females. Diagnosis was made by histopathology of cerebral cortex obtained by needle biopsy under general anaesthesia at 2-3 months of age except for one lamb that died at birth. In this case diagnosis was established post mortem.

Control sheep (Table 2.II) were age matched normal Southdown or New Zealand Romneys or normal heterozygotes (controls 1 and 2) of the South Hampshire inbred flock.

TABLE 2.I

LIST OF AFFECTED SHEEP USED IN THIS STUDY

Case No.	Sex	Age at death	Brain weight(gm)	Microscopy
1	-	mid term foetus (D)	-	Light,F,EM
2	-	mid term foetus (D)	-	Light,F,EM
3	-	mid term foetus (D)	-	Light,F,EM
4	Female	full term foetus (D)	45.6	Light,F,EM
5	Male	2.5 months (E)	74.2	Light,F,EM
6	Female	4 months (E)	76.4	Light,F,EM
7	Female	5 months (E)	70.0	Light,F,EM
8	Male	6 months (E)	73.0	Light,F,EM
9	Male	7 months (E)	66.0	Light,F,EM
10	Male	9 months (E)	65.9	Light,F,EM
11	Female	12 months (E)	68.4	Light,F,EM
12	-	14 months (E)	57.0	- - -
13	-	14 months (E)	61.0	- - -
14	Female	18 months (E)	52.0	Light,F, -
15	Male	22 months (E)	52.0	Light,F,EM
16	Female	23 months (E)	47.0	Light,F,EM
17	Female	23 months (E)	49.6	- - -
18	Male	24 months (E)	54.3	- - -
19	Female	25 months (E)	52.0	Light,F, -

D - Died; E - Euthanasia; F - Fluorescent; EM - Electron microscopy

TABLE 2.II

LIST OF NORMAL CONTROL SHEEP USED IN THIS STUDY

Case No.	Sex	Age at death	Brain weight(gm)	Microscopy
1	Female	full term foetus (D)	47.6	Light,F,EM
2	Female	3 weeks (D)	66.0	- - -
3	Male	2.5 months (E)	72.0	Light,F,EM
4	Male	4 months (E)	80.6	Light,F,EM
5	Female	5 months (E)	79.2	Light,F,EM
6	Female	6 months (E)	93.0	Light,F,EM
7	Male	9 months (E)	84.0	Light,F,EM
8	Female	12 months (E)	90.8	Light,F,EM
9	Female	22 months (D)	94.0	Light,F, -
10	-	27 months (E)	99.0	Light,F, -
11	Male	20 years (D)	-	Light,F, -

D - Died; E - Euthanasia; F - Fluorescent; EM - Electron microscopy

Preparation of tissues for gross pathology

1. Brain weight

Whole brain was removed at autopsy and weighed.

2. Gross anatomy

After midsagital sectioning, one hemisphere was fixed in 10% formol saline for a minimum of 7 days. Serial, 3-5 mm thick coronal sections were then made and examined grossly.

Selected paraffin sections from the coronal slices were stained by luxol fast blue (LFB) and haematoxylin and eosin (H & E) (Culling, 1974).

III. RESULTS

At birth, no disease specific gross changes of brain are observed. After the age of 4 to 5 months, brain atrophy and thickening of the skull bones overlying the brain are noted. These changes become more obvious with advancing age (Fig. 2.1). At terminal disease the brain is strikingly atrophic weighing 55% those of normal controls. Atrophy most affects the cerebrum (Fig. 2.2). The atrophic brain is firmer than normal. Coronal slices of the cerebrum show marked thinning of the cortex, mild atrophy of the diencephalon, moderate reduction in size of the subcortical white matter, severe attenuation of the corpus callosum and slight enlargement of the lateral ventricles (Fig. 2.3). These changes are particularly well seen in paraffin sections stained by luxol fast blue and haematoxylin & eosin (Fig. 2.4). By terminal disease, yellow discolouration of the gray matter is also noted in the coronal slices of the cerebrum.

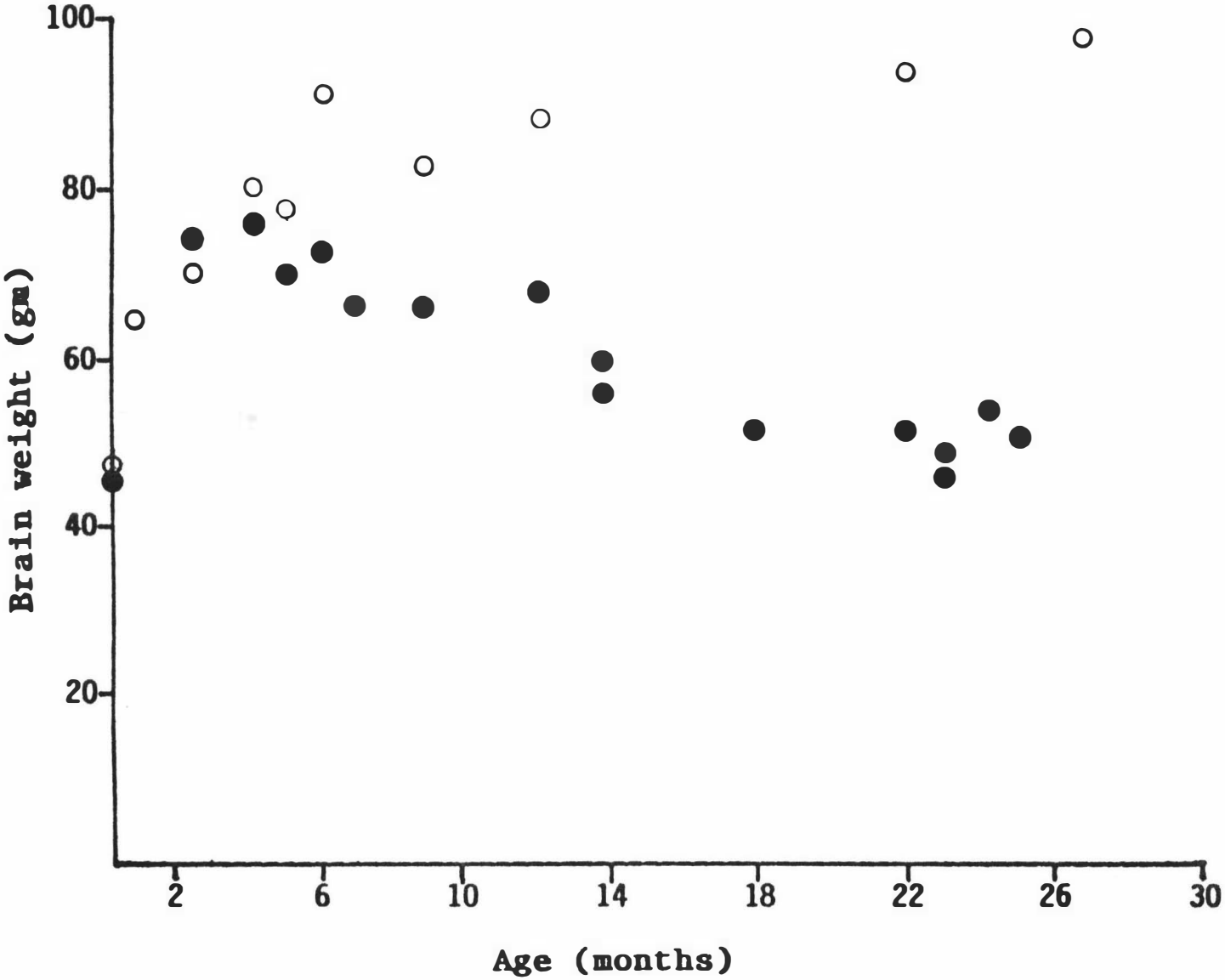
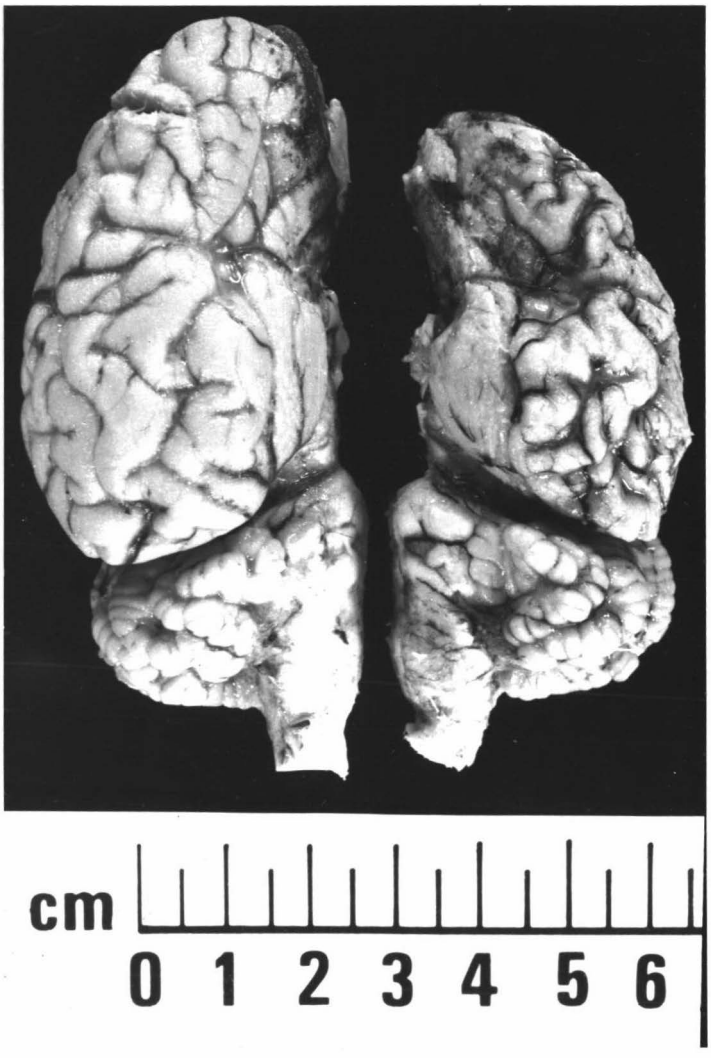


Figure 2.1 Brain weights of sheep with ceroid-lipofuscinosis (●) and age matched normal controls (○) relative to each other and age.

Figure 2.2: Lateral view of brains from a 25 months old affected sheep (right) and from a 27 months old normal sheep (left). The greatest degree of atrophy involves the cerebrum. Note marked thinning of the gyri of the cerebrum of the affected animal. (Formalin fixed.)

Figure 2.3: Transverse sections of the cerebrum from a 25 months old affected sheep (left) and from a 27 months old normal sheep (right). Note reduced size of the cerebrum, marked thinning of the cortex, severely attenuated corpus callosum and slight enlargement of the lateral ventricle in the affected sheep. Mild atrophy of the diencephalon and moderate reduction in size of the subcortical white matter are also noted in the affected sheep. (Formalin fixed.)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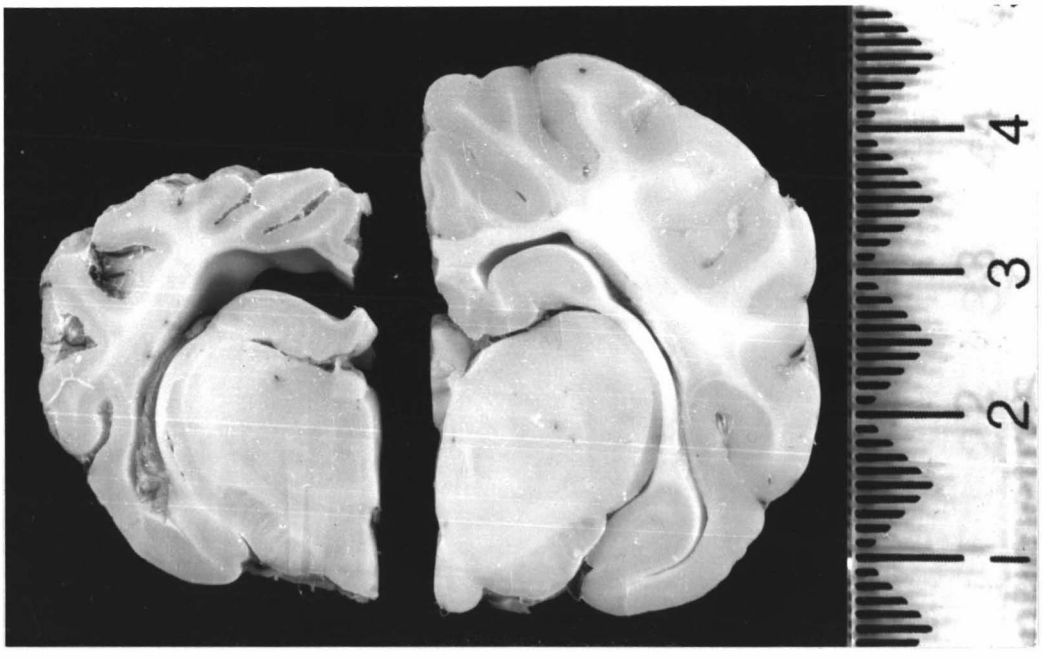
0 1 2 3 4 5 6


1 2 3 4

IV. DISCUSSION

The most striking gross pathological change in affected sheep is the brain atrophy. This affects mainly the cerebrum and to a lesser extent, the diencephalon. The cerebellum and spinal cord are largely spared. This contrasts with the brain atrophy in some types of human ceroid-lipofuscinosis and in canine ceroid-lipofuscinosis. In the infantile and late infantile types of human syndrome, and in the canine syndrome, the atrophy affects both the cerebrum and cerebellum (Koppang, 1973/74; Zeman, 1976).

In affected sheep, the brain atrophy is noted by the age of 4 to 5 months and it becomes more obvious with advancing age. By terminal disease, at around 24 months of age, the brain weights of affected sheep are 55% of those of normal sheep. On the other hand, brain atrophy in the canine syndrome is not recorded until 12 months of age. Brain weights of affected dogs are about 70% of those of normal controls at terminal disease, i.e., at the age of 20 to 25 months (Koppang, 1973/74).

The firm consistency of the affected brain is expected to be due to the intense fibrillary astrogliosis noted by light microscopy (see Chapter III). Yellow discolouration of the grey matter in the cerebrum reported in the human and canine syndromes is also noted in ovine cases.

CHAPTER III

LIGHT MICROSCOPY

I. INTRODUCTION

Progressive accumulation of autofluorescent lipopigments within neurones and a wide variety of other cell types is the characteristic histological feature of the ceroid-lipofuscinoses (Zeman, 1976). Associated changes are mainly limited to the central nervous system, and consist of neuronal loss, astrocytosis and infiltration by macrophages. Subsequent degeneration of nerve fibres in the white matter has also been reported in humans (Zeman et al., 1970; Haltia et al., 1973b) and in the dog (Koppang, 1973/74; Rac & Giesecke, 1975).

This chapter concerns a longitudinal light microscopic study whose purpose was to describe the sequential and topographical changes in the brain of sheep with ceroid-lipofuscinosis.

II. MATERIALS AND METHODS

Animals

In addition to the animals used in the study on gross pathology, three affected mid term foetuses were used (Tables 2.I and 2.II). Two of these were obligate affected individuals and were obtained by caesarean section from homozygously affected 12 months old ewes which had been mated with homozygous males (experimental cases 1 and 2). The third was taken from an affected 22 months old ewe when she was euthanazed in the later stages of the disease (experimental case 3). She was presumed to be mated by an affected male.

Preparation of tissues for microscopy

1. Light microscopy

Fixed coronal sections of the whole brain, ranging from the frontal lobe to the medulla oblongata, and the spinal cord at the level of C_1 were processed by routine procedures for paraffin embedding and sectioning. Deparaffined sections were stained by haematoxylin and eosin (H & E), cresyl echt violet, periodic acid-Schiff (PAS), luxol fast blue (LFB) (Culling, 1974) and Sudan black B (Disbrey & Rack, 1970). Selected sections were also stained by the long Ziehl-Neelsen method (x3 h), Schmorl's ferric ferricyanide method (Bancroft & Stevens, 1982), Holmes' silver & luxol fast blue (Culling, 1974) and Marchi's method for degenerated myelin (Culling, 1974).

Foetal brain was treated with the same process for fixation, paraffin embedding and sectioning as above. Deparaffined sections were stained by haematoxylin and eosin (H & E), periodic acid-Schiff (PAS), luxol fast blue (LFB), Sudan black B, the long Ziehl-Neelsen method (x3 h) and Schmorl's ferric ferricyanide method.

The immunocytochemical study was performed on brain tissue of affected sheep (experimental cases 4-6, 10, 11, 14, 16 and 19) and age matched normal sheep (control cases 1, 3-5 and 7-10) to demonstrate glial fibrillary acidic protein (GFAP) of astrocytes (Hsu & Raine, 1981; Bonnard et al., 1984). Paraffin sections cut at 10 µm were routinely processed through xylene and graded alcohol and treated with 1% normal bovine serum (Sigma) for 5 min. The tissues were then treated with rabbit anti-GFAP IgG (Dakopatts, Denmark) (1:750) for 3 h, donkey anti-rabbit IgG (Amersham International plc, Amersham, UK) (1:200) for 30 min and streptavidin-biotin-peroxidase complexkt (Amersham International plc, Amersham, UK) (1:200) for 15 min. Each stage was followed by washing (x3) in 0.01M phosphate

buffer. The application of diaminobenzidine-H_2O_2 (DAB-H_2O_2) for 5 min allowed the reaction product to be seen. Sections were lightly stained with haematoxylin as a counterstain. Method-specific controls were performed by omitting the primary antibody.

Sections of 0.5 to 1.0 µm in thickness were cut for light microscopy from epoxy resin embedded tissues prepared for electronmicroscopy (see Chapter IV). These sections were stained by 1% toluidine blue in 0.1M phosphate buffer (pH 7.2) on a hot plate at 80°C for 45 s.

2. Fluorescent microscopy

To demonstrate autofluorescence, unstained deparaffined sections were viewed under ultraviolet light in an Olympus microscope BHS. The microscope was fitted with a 100W high pressure mercury burner and halogen lamp, using exciter filter U(UG-1) whose applicable excitation regions were 334 to 365 nm and barrier filter L-420 which blocked wavelengths shorter than 420 nm.

III. RESULTS

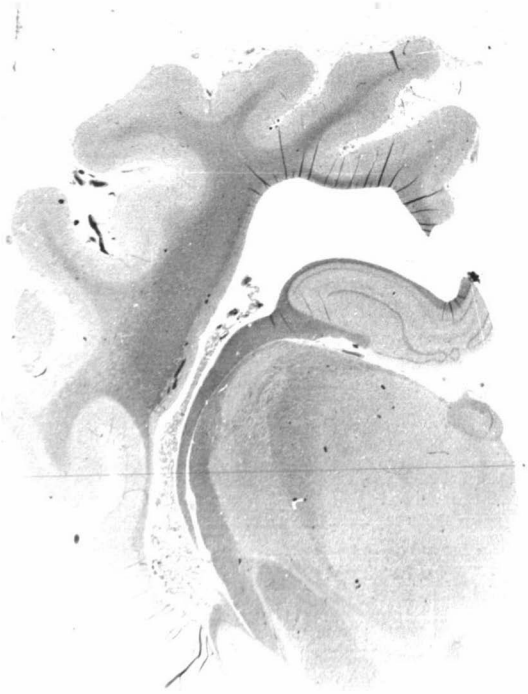
Accumulation of pigment granules

The disease is characterized by the progressive accumulation of autofluorescent granules in neuronal perikarya (Fig. 3.1). Glial and endothelial cells are similarly involved, but to a lesser degree.

The granules stain intensely black with Sudan black B (Fig. 3.2a), deep blue with luxol fast blue (Fig. 3.2b), are moderately PAS-positive (Fig. 3.2c) and slightly acid-fast. They do not stain with Schmorl's stain. In general, the

Figure 2.4: Transverse section of the cerebrum of a 25 months old affected sheep. Changes described in Fig. 2.3 are well demonstrated. Note myelin loss in the subcortical white matter. (Paraffin section, luxol fast blue and H & E x3)

Figure 3.1: Ventral horn cells of the cervical spinal cord of a 23 months old affected sheep. Note clusters of autofluorescent granules in the neuronal perikarya. (Unstained deparaffined section viewed under ultraviolet light, x600)



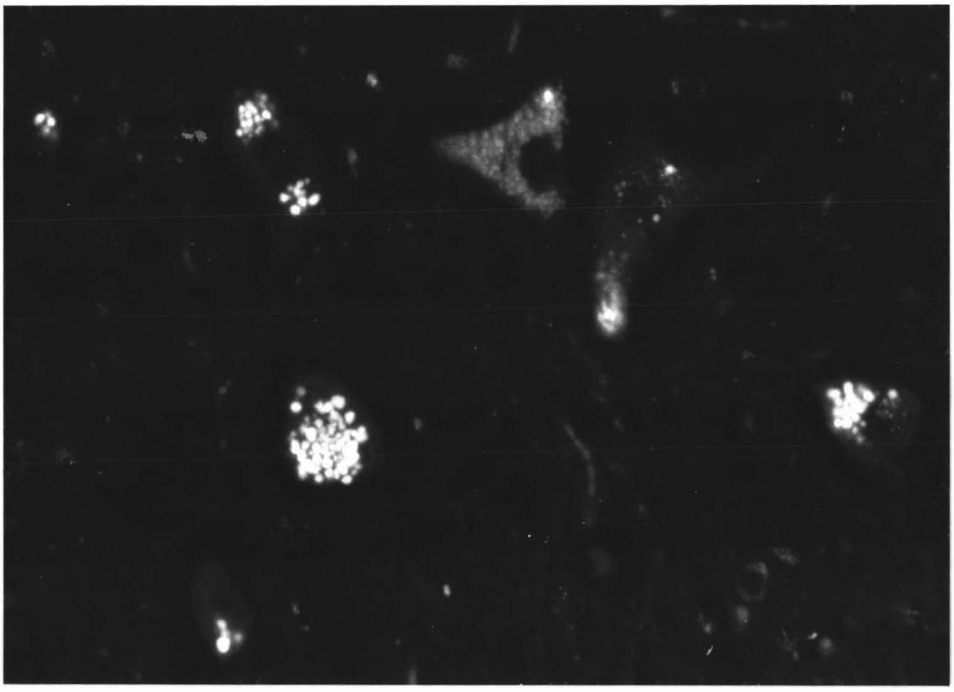
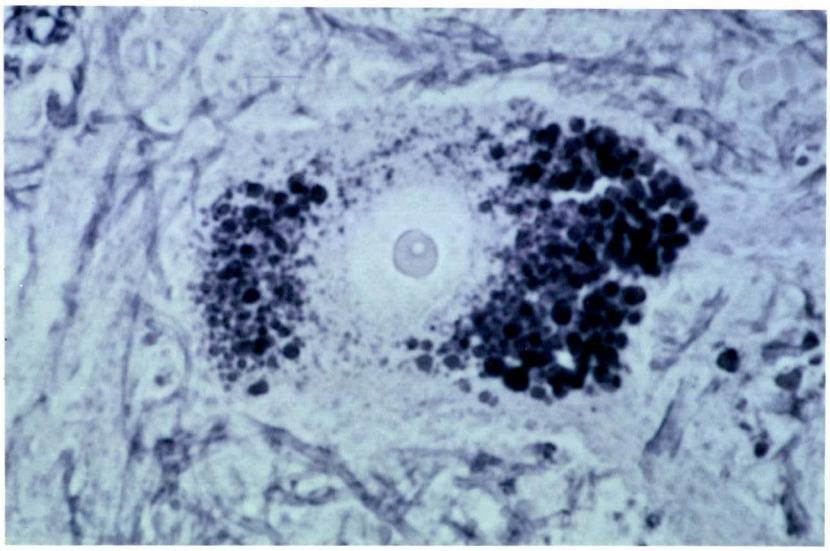
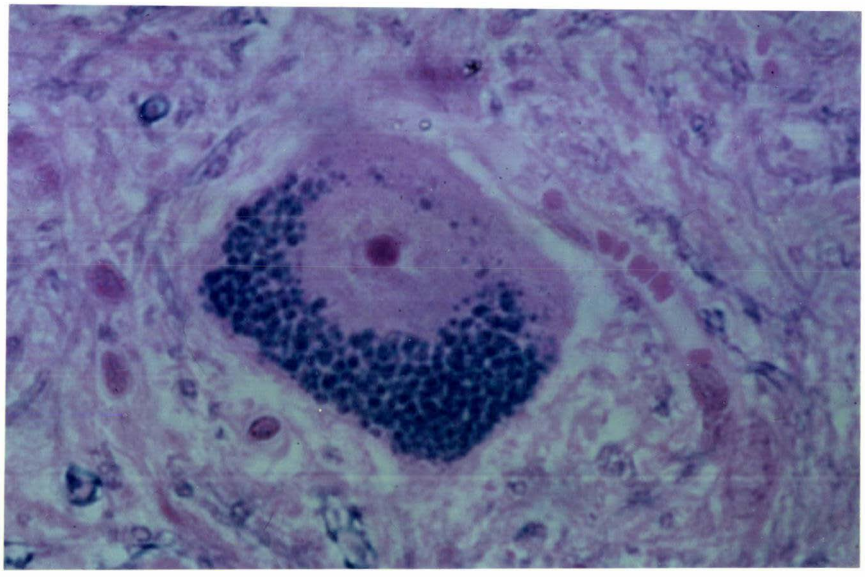


Figure 3.2a: Ventral horn cell of a 23 months old affected sheep. Lipopigment granules accumulating in the cytoplasm stain black with Sudan black B. (Paraffin section, Sudan black B x650)

Figure 3.2b: Ventral horn cell of a 23 months old affected sheep. Lipopigment granules filling the cytoplasm stain blue with luxol fast blue. (Paraffin section, luxol fast blue and H & E x650)

Figure 3.2c: Ventral horn cell of a 23 months old affected sheep. Lipopigment granules filling the cytoplasm stain red with PAS. (Paraffin section, periodic acid-Shiff x650)





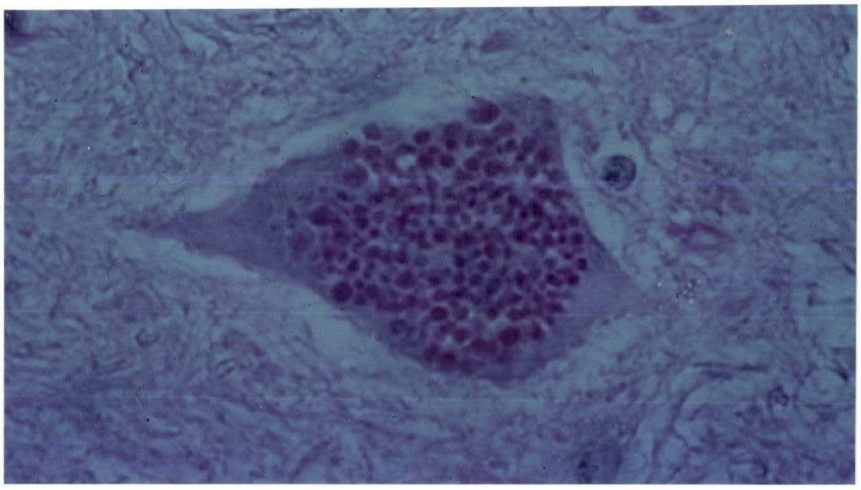
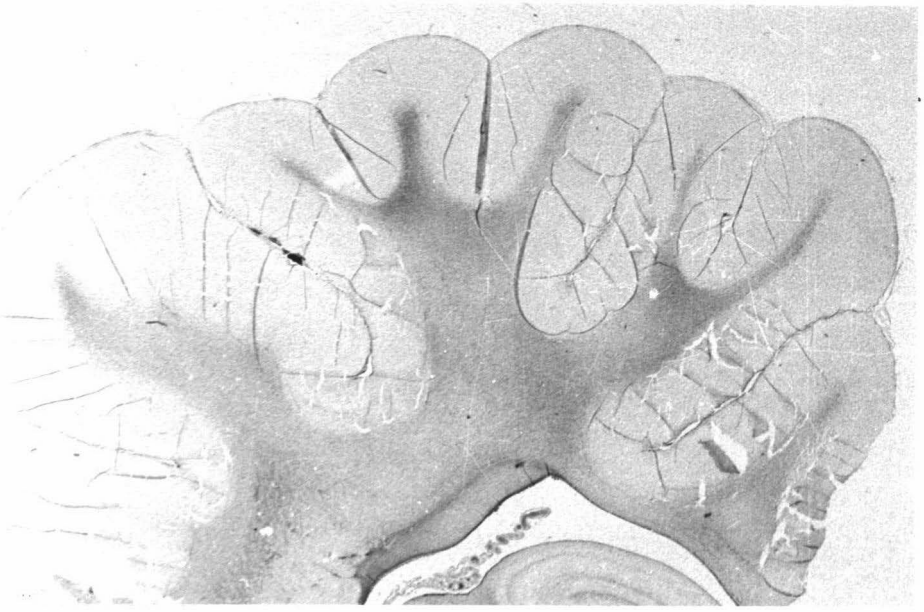


Figure 3.11: A coronal section of the parietal lobe of the cerebrum in a 5 months old normal lamb (a) is compared with similar sections of a 5 months (b) and a 18 months old (c) affected lambs. The immunocytochemical stain for glial fibrillary acidic protein demonstrates astrocytosis in the middle area of the isocortex with a clear-cut dark band (arrows). This occurs first in the dorsal gyri (b) extending into the lateral gyri with increased age (c). Concomitant with this is atrophy of the same areas. Astrocytosis of the white matter is also prominent. (Paraffin sections, immunocytochemical method for glial fibrillary acidic protein of astroglia x4)

a



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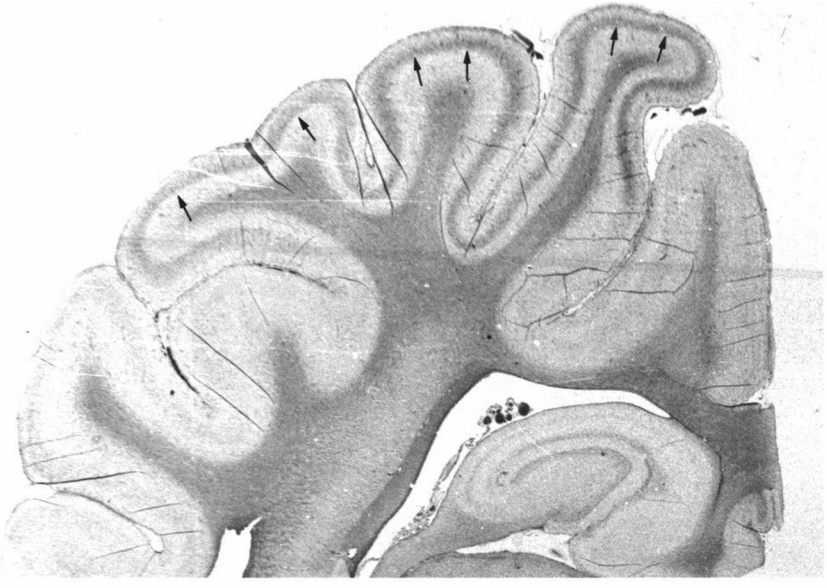

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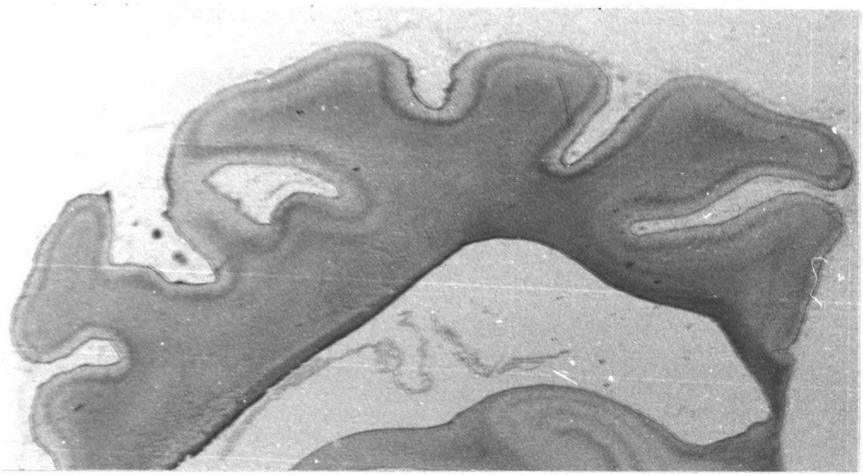
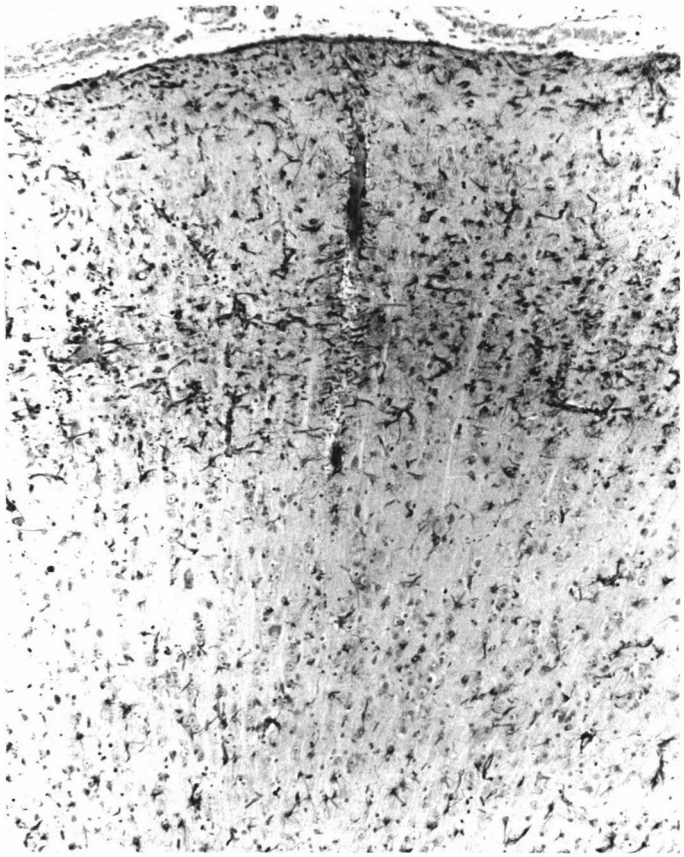
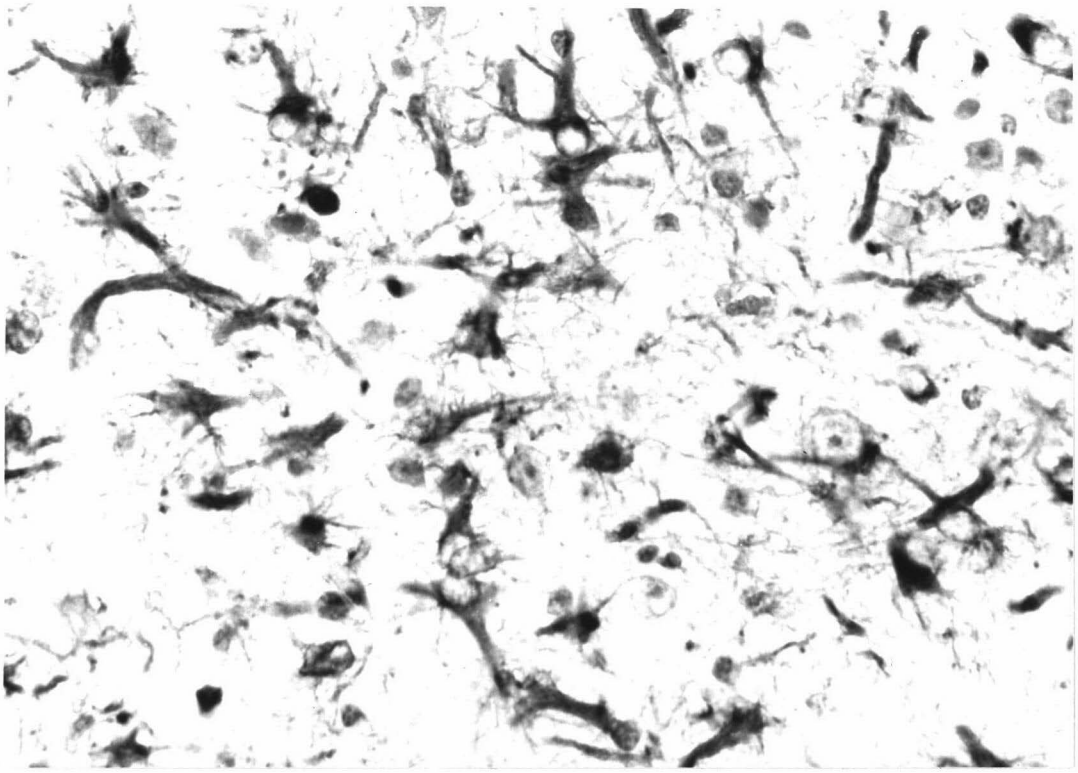


Figure 3.12: Medium power magnification of the cerebral cortex from the same section of 5 months old affected lamb as shown in Fig. 3.11. It confirms that the dark band interpreted as an astrocytosis does consist of cellular and fibrous components. (Paraffin section, immunocytochemical method for glial fibrillary acidic protein of astroglia x80)

Figure 3.13: High power magnification of the dark band as shown in Figs. 3.11 & 3.12. Note hyperplastic and hypertrophic astrocytes with prominent fibrillary processes. (Paraffin sections, immunocytochemical method for glial fibrillary acidic protein of astroglia x600)





differences mentioned in neuronal granule accumulation among cell types, there is no apparent difference in quantitative distribution between the topographical areas.

Grey matter

Although pigment granules are present in mid term and full term foetal brains, there are no secondary changes in the grey matter. However, cytoarchitectural changes in the grey matter are present by 2.5 months of age. These are composed of a mild degree of neuronal loss, fibrillary astrogliosis, and macrophage reaction. These changes become more severe with advancing age. They occur first and are most severe in the telencephalic isocortex. This is followed by the moderately severe changes in nuclei in outer areas of the hypothalamus and the anterior colliculus of the mescencephalon. The changes in the hippocampus, basal ganglia, thalamus and subthalamus occur later in the disease and are relatively mild, although neurones in these regions may be distended with cytoplasmic deposits by terminal disease (Tables 3.II and 3.III). The cerebellum, spinal cord, pons and medulla oblongata appear to be relatively spared secondary degenerative changes.

In the telencephalon, the changes occur first and are most severe in the parietal lobe. This is followed by the less severe changes in the occipital and frontal lobes. Mild changes occur in the temporal lobe later in the disease. Neuronal loss appears to occur first and be most severe in the middle area of the isocortex, showing laminar distribution (Fig. 3.8). It gradually extends into the other areas until terminal disease, when there is a marked neuronal loss throughout the whole isocortex (Fig. 3.8). However, some neurones remain. These are mainly the larger pyramidal cells in the lower area of the isocortex. They harbour large amounts of granular deposits in their cytoplasm and their dendrites and axons often appear

TABLE 3.II

EVOLUTION OF HISTOLOGICAL CHANGES ACCORDING TO AGE IN BRAINS OF SHEEP AFFECTED WITH CEROID-LIPOFUSCINOSIS

Case number	1	4	5	7	11	14	19
Age	Mid term foetus	Full term foetus	2.5months	5months	12months	18months	25months
Cerebral cortex							
Neuronal storage	+	+	++	++	+++	++++	++++
Loss of neurones	-	-	+	++	+++	+++	++++
Number of phagocytes	+	+	+	++	+++	+++	++++
Astrogliosis	-	-	+	++	+++	+++	++++
Subcortical white matter							
Wallerian type degeneration	-	-	+	++	+++	+++	++++
Number of phagocytes	-	-	+	+	+	+	++
Astrogliosis	-	-	+	+	++	++	+++

- = not present; + = minimum; ++ = mild; +++ = moderate; ++++ = marked.

TABLE 3.III

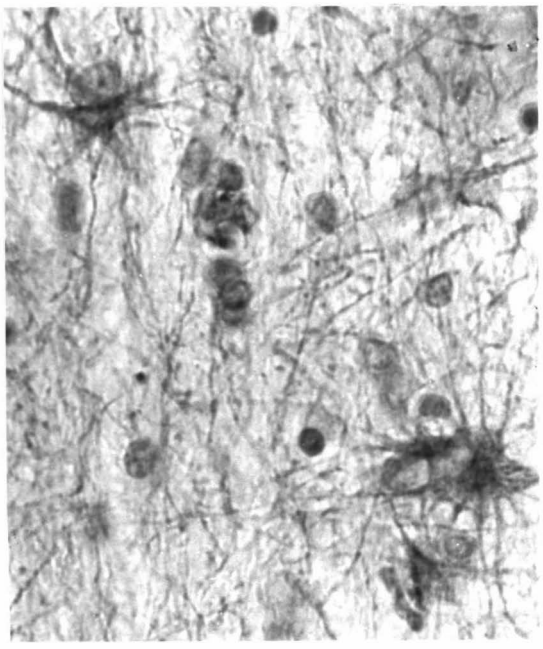
RELATIVE DISTRIBUTION AND SEVERERITY OF LESIONS IN THE SELECTED AREAS OF THE CENTRAL NERVOUS SYSTEM OF 12 TO 25 MONTHS OLD SHEEP AFFECTED WITH CEROID-LIPOFUSCINOSIS

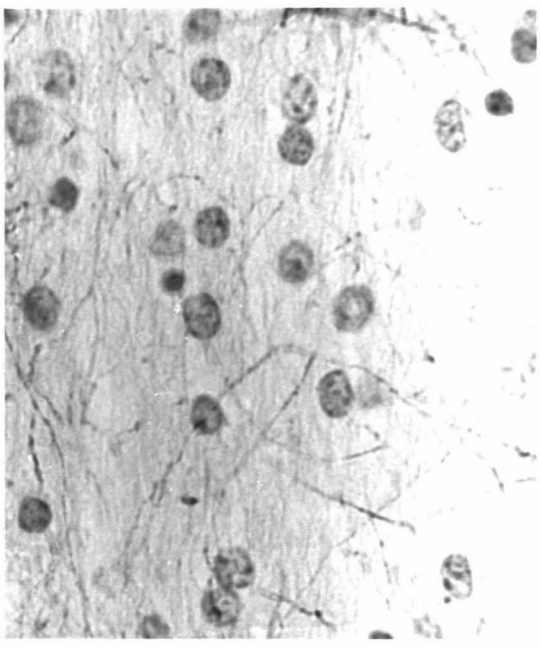
	Neuronal loss	Macrophages	Astrogliosis
Frontal cortex	+++	++	+++
Parietal cortex	++++	+++	++++
Temporal cortex	++	+	++
Occipital cortex	+++	++	+++
Basal ganglia	+	+	+
Thalamus	+	+	+
Hypothalamus	+++	++	++
Hippocampus	+	+	+
Anterior colliculus	+++	++	++
Olivary nucleus	-	-	-
Cerebellum	-	-	-
Spinal cord	-	-	-

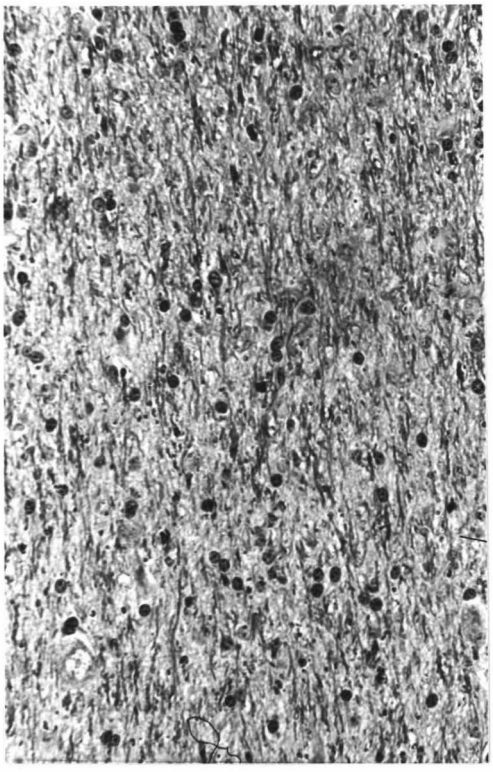
- = not present; + = minimum; ++ = mild; +++ = moderate; ++++ = marked.

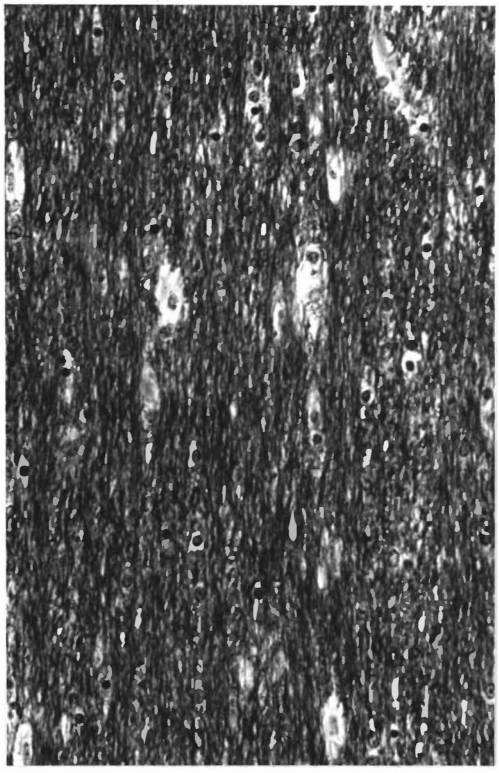
Figure 3.18: Subcortical white matters of a 5 months old affected lamb (left) and of an age matched normal lamb (right). Note the increased fibrillary astrocytic processes in the affected animal. (Paraffin sections, immunocytochemical method for glial fibrillary acidic protein of astroglia x700)

Figure 3.19: Subcortical white matters of a 23 months old affected sheep (left) and of an age matched normal sheep (right). Note a marked loss of myelin in the affected animal. (Paraffin sections, luxol fast blue and H & E x300)









White matter

Changes in the white matter consist of Wallerian type degeneration and accompanying fibrillary astrogliosis. The macrophage reaction is comparatively mild compared with that in the grey matter.

Wallerian type degeneration is identified by the presence of myelin ovoids in the subcortical white matter as early as 2.5 months of age. By this stage fibrillary astrogliosis of a mild degree is also noted as are a few granule-laden macrophages. These changes become more obvious with advancing age (Table 3.IV).

In the late stages, vacuolar spaces with and without phagocytes and cell debris within them, which are interpreted as evidence of Wallerian type degeneration, are noted diffusely in the subcortical white matter and internal capsule (Figs. 3.16 & 3.17). This is accompanied by pronounced fibrillary astrogliosis (Fig. 3.18). There is a marked loss of myelin in the subcortical white matter by terminal disease (Fig. 19). The hyperplastic and hypertrophic astrocytes harbour pigment granules similar to those in neurones, but to a lesser degree. Clumps of macrophages whose cytoplasm is distended with the granular deposits are occasionally noted within the white matter, particularly around blood vessels.

The distribution and degree of the changes in the subcortical white matter appear to parallel those of the neuronal destruction in the telencephalic isocortex of the various lobes. The change is most severe beneath the parietal lobe cortex, less severe beneath the occipital and frontal lobe cortices, and relatively mild beneath the temporal lobe cortex. After 12 months of age, the Wallerian type degeneration is seen to be more extensive in the brain. It appears to extend

TABLE 3.IV

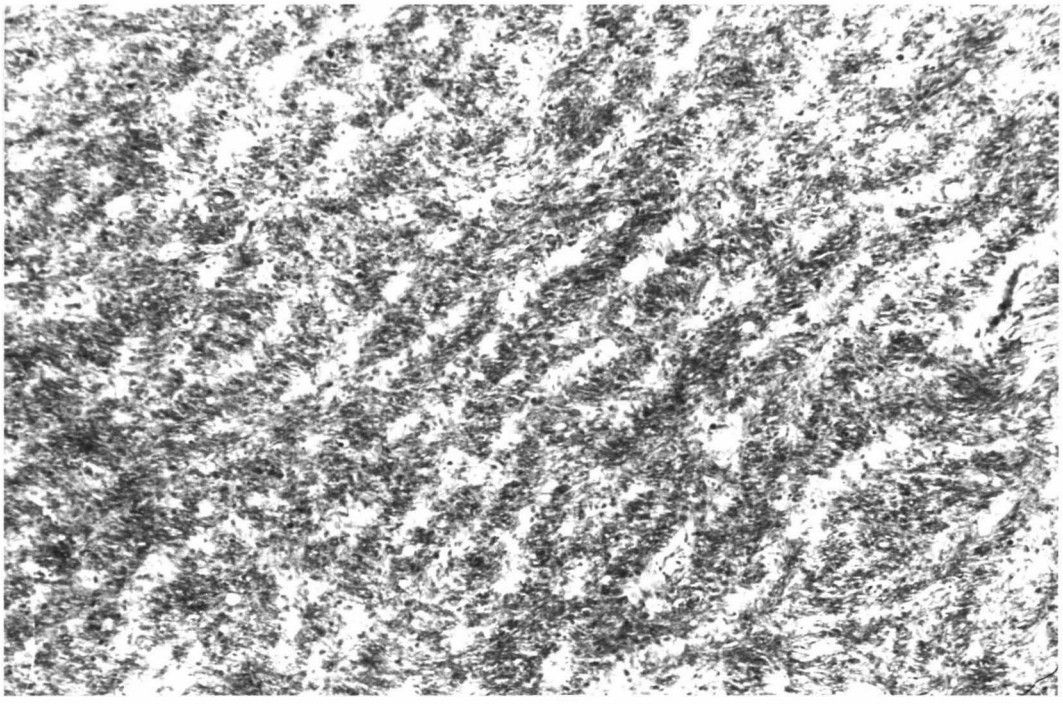
DISTRIBUTION AND EVOLUTION OF WALLERIAN TYPE DEGENERATION IN BRAINS OF SHEEP AFFECTED WITH CEROID-LIPOFUSCINOSIS

Case number	4	5	7	11	14	19
Age	Full term foetus	2.5months	5months	12months	18months	25months
Subcortical white matter	-	+	++	+++	+++	+++
Internal capsule	-	-	-	+	+	++
Crus cerebri	-	-	-	-	+	++
Medullary pyramid	-	-	-	-	+	++
Optic tract	-	-	-	-	-	++

- = not present; + = minimum; ++ = mild; +++ = moderate; +++++ = marked.

Figure 3.14: Cerebral cortex of a 23 months old affected sheep. Hypertrophic astrocytes with bizarre nuclei contain lipopigment granules (arrows) in the cytoplasm. (Paraffin section, luxol fast blue and H & E x800)

Figure 3.15: Higher power magnification of a perivascular cuff of lipid laden macrophages in the cerebral cortex of a 23 months old affected sheep. Note considerable heterogeneity in the staining of the granular deposits in the cytoplasm. (Paraffin section, luxol fast blue x650)



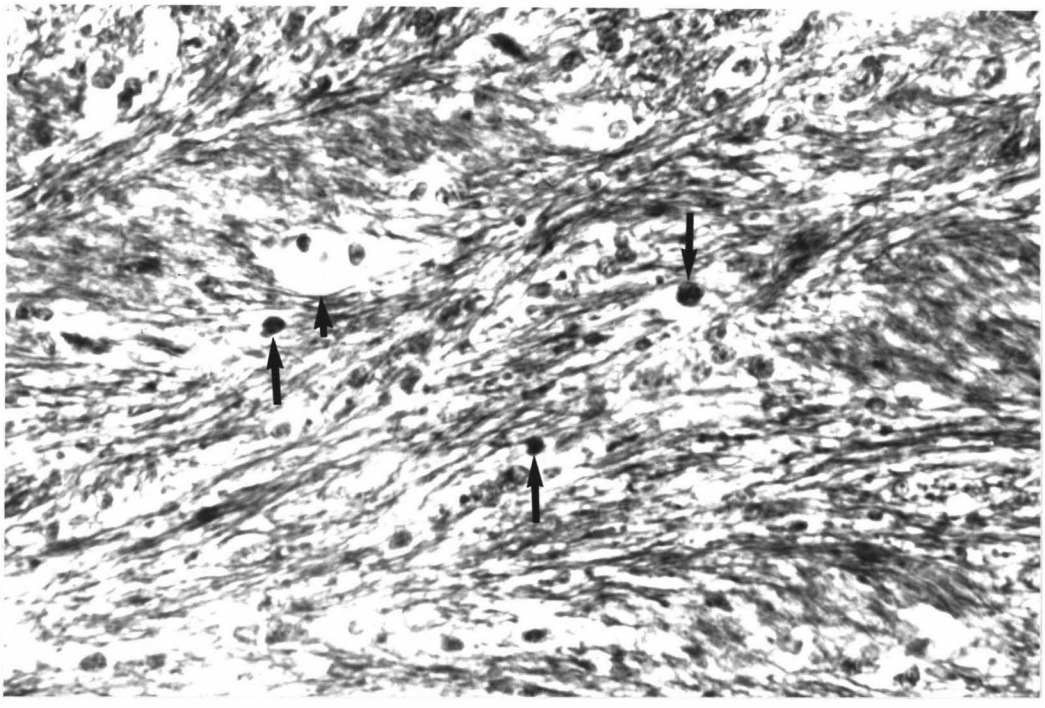
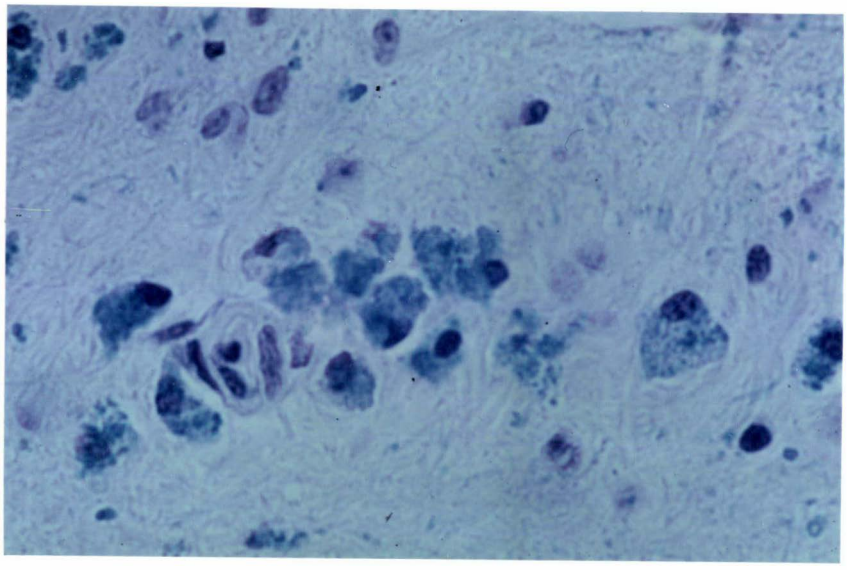


Figure 3.16: Internal capsule of a 23 months old affected sheep. Many vacuolar spaces are diffusely seen in the white matter. (Paraffin section, luxol fast blue and H & E x100)

Figure 3.17: Higher power magnification of one part of Fig. 3.16. A phagocyte with cell debris in its cytoplasm is present in a vacuolar space (short arrow). Note many myelin ovoids (long arrows). (Paraffin section, Holmes' silver and luxol fast blue x500)



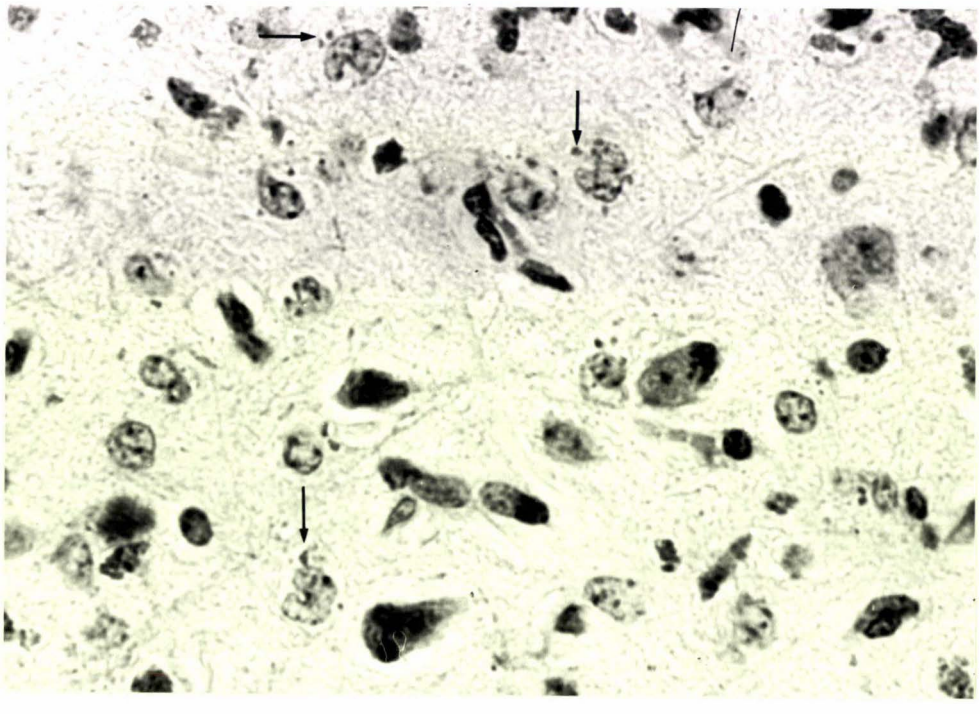
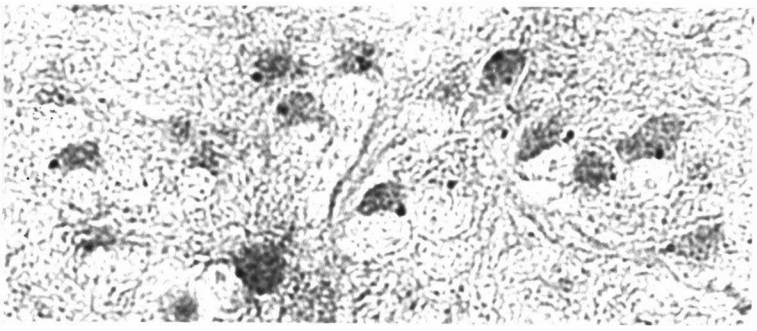
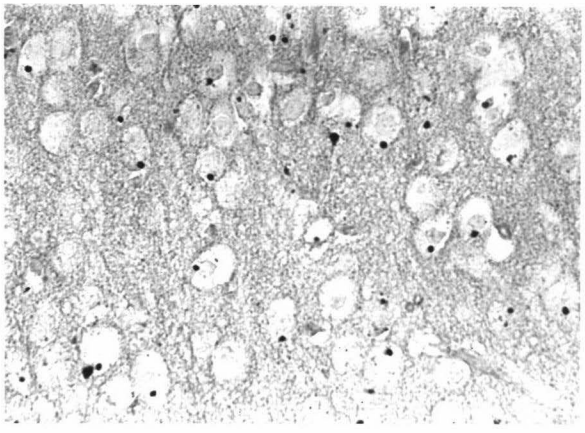


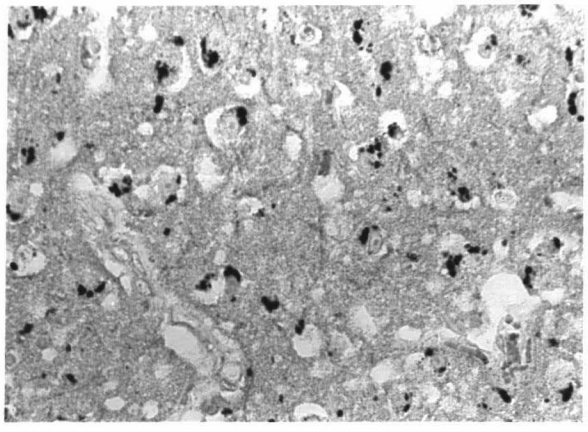
Figure 3.4: Cerebellar cortex from an obligate homozygous affected mid term foetus. Discrete Sudan black staining inclusions indicative of ceroid-lipofuscinosis are already present in Purkinje cells. (Paraffin section, Sudan black B x700)

Figure 3.5: Cerebral cortices of a full term affected foetus (a), a 5 months old affected lamb (b), a 18 months old affected lamb (c) and a 23 months old affected sheep (d). The amount of Sudanophilic deposits in neurones as well as in the cerebral cortex increases with advancing age. Large clusters of Sudanophilic deposits with variation in the staining and a neuronal loss are noted by terminal disease. (Paraffin section, Sudan black B x300)





a



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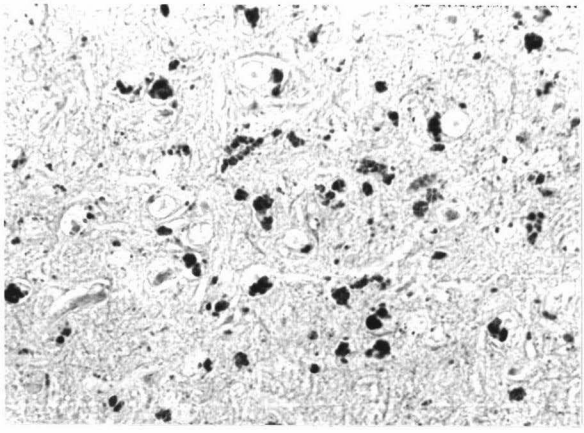

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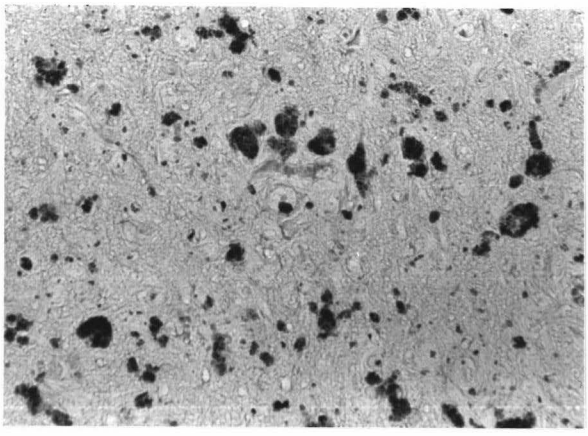
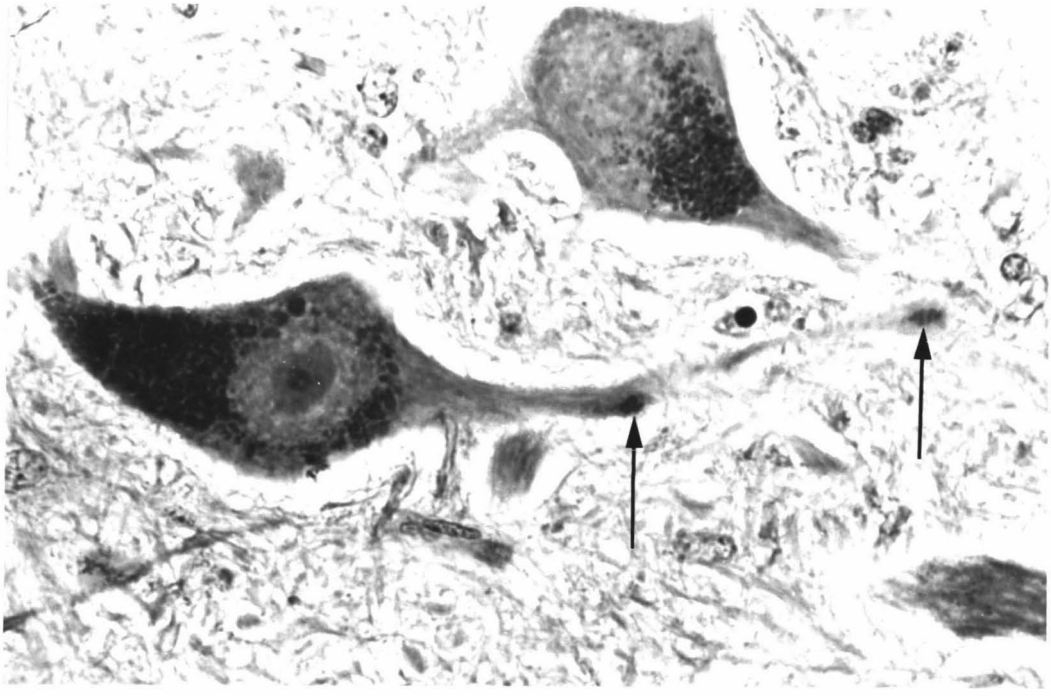


d

Figure 3.6: Ventral horn cells of a 18 months old affected lamb. Lipopigment granules accumulate in the neuronal perikarya, extending into axon hillock and even into the proximal part of the axon (arrows). (Paraffin section, luxol fast blue and H & E x600)

Figure 3.7: Cerebral cortex of a 23 months old affected sheep, showing neuronal loss and pronounced gliosis. Deposit material is mainly in macrophages (arrows). (Paraffin section, luxol fast blue x500)



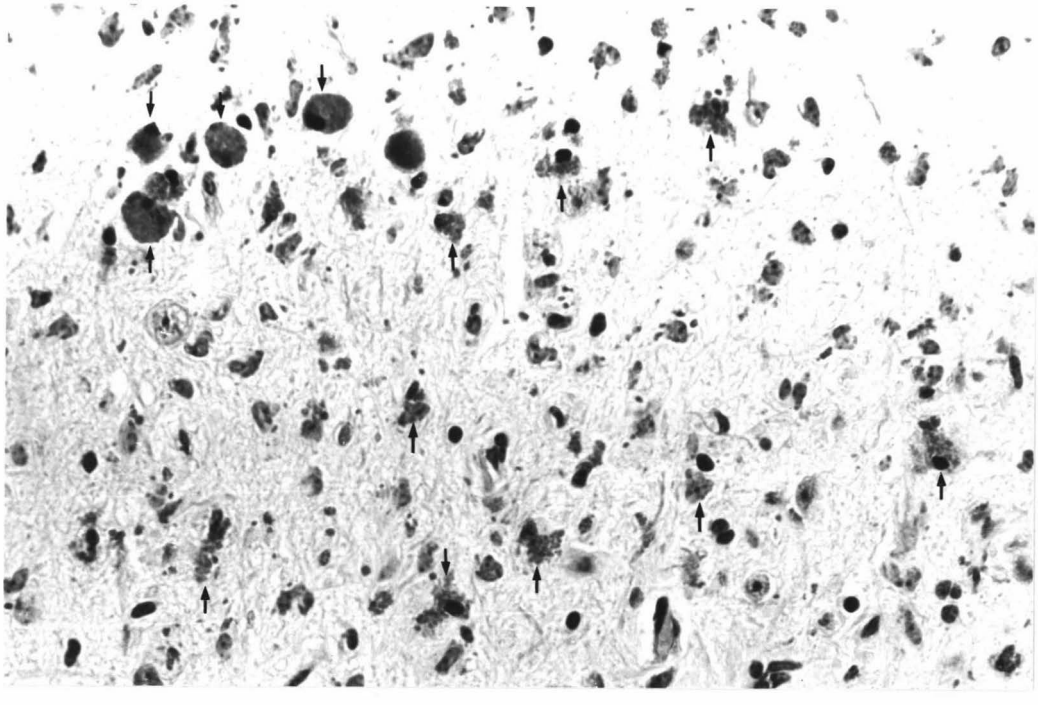
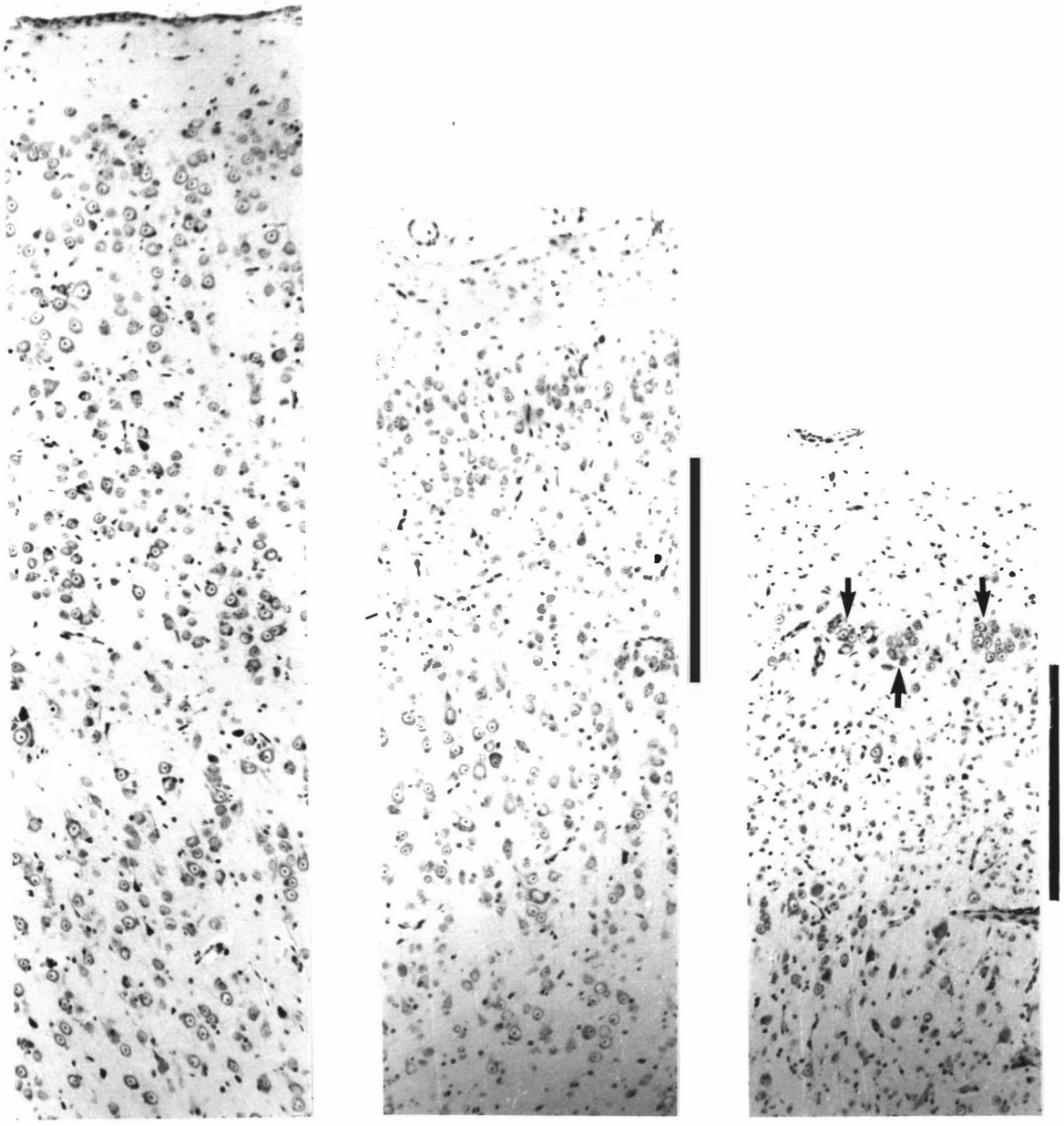


Figure 3.8: A transverse sections of the cerebral cortex of a normal 5 months old lamb (a) is compared with those of a 5 months old (b) and a 18 months old (c) affected lambs. At 5 months there is already a evidence of atrophy with particular loss of neurones in the middle area of the isocortex (vertical bar). This is exacerbated in the 18 months old animal with a general loss of neurones in other areas as well. Note the focal arrangements of remaining neurones (arrows) in the superficial area of the isocortex of the 18 months old animal. (Paraffin sections, cresyl echt violet x100)


a
b
c

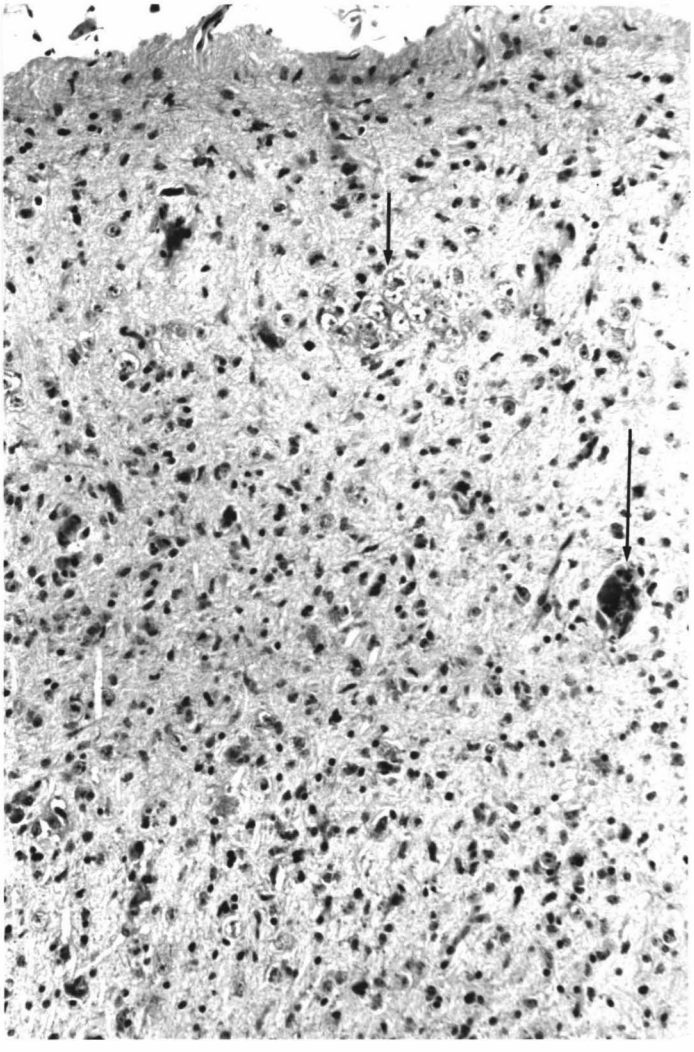
swollen. Some smaller neurones may also remain. They are either focally located in the superficial area associated with occasional glial components in the foci (Figs. 3.8, 3.9 & 3.10), or diffusely in the other areas. The foci of remaining neurones, which are obvious by 9 months of age, become progressively more prominent up until 18 months of age. After that age they become less obvious as these neurones also undergo necrosis.

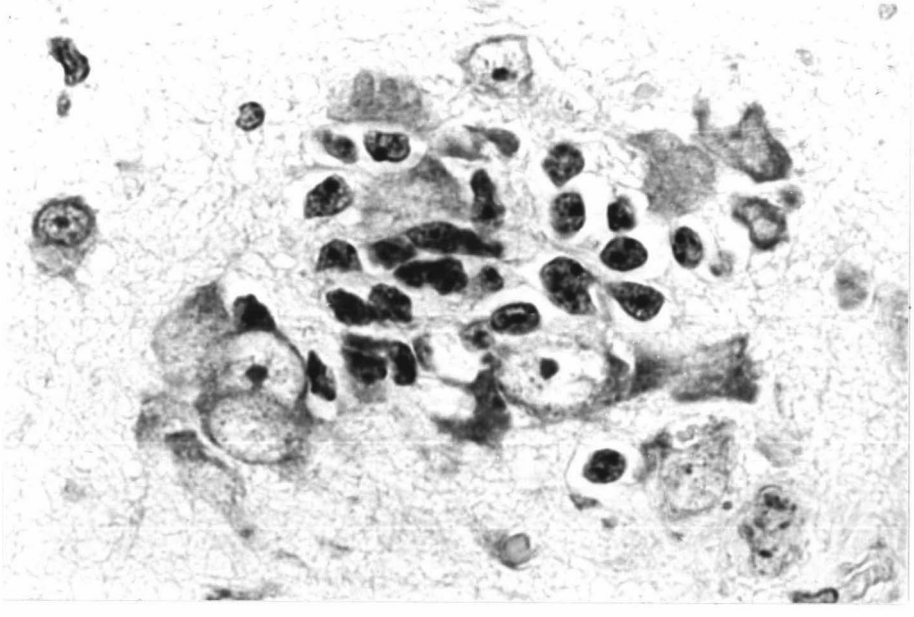
The fibrillary astrogliosis is well demonstrated in paraffin sections treated with immunocytochemical method for glial fibrillary acidic protein. The change appears to affect the dorsal part of the cerebrum more intensely than other parts, although it extends over the whole cerebrum by terminal disease (Fig. 3.11). The change occurs first in the middle area of the isocortex. This becomes more obvious with advancing age, presenting a clear-cut band of an intense network of fibrillary astroglia (Figs. 3.11, 3,12 & 3.13). This distribution pattern parallels that of the progressive neuronal degeneration. The fibrillary astrocytes are both hyperplastic and hypertrophic. They show enlarged nuclei of bizarre shape and their processes in which a few storage granules similar to those in the neurones are present are thickened (Fig. 3.14). Prominent capillaries are noted in the astrocytic network.

The number of macrophages in the affected areas increases with advancing neuronal destruction (Fig. 3.7). They tend to have small, rounded and hyperchromatic nuclei. Their cytoplasm is distended with the granular deposits. By terminal disease, these macrophages form cuffs around blood vessels where the granular deposits in the cytoplasm exhibit considerable heterogeneity with Sudan black B and luxol fast blue stains (Fig. 3.15).

Figure 3.9: Cerebral cortex of a 23 months old affected sheep, showing a marked neuronal loss and a prominent gliosis. Note the focal arrangement of remaining neurones (short arrow) in the superficial area of the isocortex and a cluster of lipid laden cells (long arrow) interpreted as macrophages. (Paraffin section, luxol fast blue and H & E x200)

Figure 3.10: A small glial nodule associated with a small focal arrangement of remaining neurones in the superficial area of the cerebral cortex from a 23 months old affected sheep. (Paraffin section, cresyl echt violet x600)





topographically from the subcortical white matter through areas in the corticospinal system, such as internal capsule, crus cerebri of the mescencephalon, corticospinal tract in the pons, into the medullary pyramid in the medulla oblongata (Table 3.IV). In the corticospinal system, however, changes are mild and the spinal cord is spared. At terminal disease myelin ovoids are noted in the optic tract.

Cytoplasmic pigment granules (lipofuscin) in normal sheep

Cytoplasmic pigment granules are also noted in neurones and glial cells in brain of normal sheep. However, the size and amount of these granules are considerably smaller than those of the pigment granules in sheep with ceroid-lipofuscinosis, reaching up to only approximately 2 µm in diameter by 20 years of age. Differences in staining characteristics between the pigment granules of affected sheep and those of normal sheep are also present (Table 3.V). The cytoplasmic lipofuscin granules in normal sheep do not stain with luxol fast blue. They appear to be slightly stained with Sudan black B and PAS stains as very fine granules at birth. In addition to these, they may exhibit autofluorescence under ultraviolet light by 12 months of age. By 27 months of age, they are slightly positive with Schmorl's and long Ziehl-Neelsen methods and slightly yellow/brown with haematoxylin and eosin stain.

In general, the intensity of staining and autofluorescence increases as the size of the granules increases with age (Table 3.V). This is prominent in astrocytes. There are no obvious secondary changes associated with lipofuscin.

TABLE 3.V

AUTOFLUORESCENCE AND HISTOCHEMICAL REACTIONS OF NEURONAL PIGMENT GRANULES (LIPOFUSCIN) IN NORMAL SHEEP

Case number	1	3	5	8	10	11
Age	Full term foetus	2.5months	5months	12months	27months	20years
Autofluorescence	-	-	-	+	++	+++
Sudan black B	+	+	+	+	++	+++
Luxol fast blue	-	-	-	-	-	-
PAS	++	++	++	++	+++	+++
Long Z.N.	-	-	-	-	+	++
Schmorl's	-	-	-	-	+	++
H & E	-	-	-	-	+	+++

PAS = Periodic acid-Shiff; Long Z.N. = long Ziehl-Neelsen method; Schmorl's = Schmorl's ferric ferricyanide method; H & E = haematoxylin and eosin; - = no reaction; + = weakly positive; ++ = moderately positive; +++ = strongly positive.

IV. DISCUSSION

The histochemical characteristics of stored pigment granules in affected sheep are essentially the same as those in the analogous human syndromes. These in ovine ceroid-lipofuscinosis stain with Sudan black B and luxol fast blue. They are also slightly acid-fast, moderately PAS-positive and autofluorescent in ultraviolet light. The infantile form of human ceroid-lipofuscinosis differs from this and other forms in as much as its cytosomes do not stain with luxol fast blue (Lake, 1984). In general, the intensity of staining and autofluorescence increases as the size of the granules increases with age (Table 3.I).

Neuronal pigment granules, interpreted as age pigment (i.e. lipofuscin), are also noted in the brain of normal sheep. However, the size and amount of these pigments are considerably smaller than those of the pigment granules in affected sheep. This is also noted in the canine disease (Koppang, 1973/74). Age pigment in the brains of sheep do not stain with luxol fast blue but show yellow/brown with haematoxylin and eosin staining in aged animals (Table 3.V).

Cytoplasmic pigment granules in ovine ceroid-lipofuscinosis are detected by light microscopy in mid term and full term foetal brains. However, it was not until 2.5 months of age that lipopigments were detected by light microscopy in the brain of dog with ceroid-lipofuscinosis (Koppang, 1973/74).

A considerable effort in time and labour has gone into the study of correlating the degree of lipopigment accumulation with specific cytoarchitectonic areas in the ceroid-lipofuscinoses (Zeman & Siakotos, 1973). However, few reports are available on the presence of regional differences in

pigment accumulation in the central nervous system in both human and animal ceroid-lipofuscinoses. In canine ceroid-lipofuscinosis, pigment granules are present in the neurones of all regions of the central nervous system (Koppang, 1973/74). Although there appears to be no obvious regional differences in pigment accumulation in the forebrain of sheep with ceroid-lipofuscinosis, there is relatively little pigment in neurones of the granular layer of the cerebellum and in the inferior olivary nucleus of the medulla oblongata. This contrasts with the topographical pattern of lipofuscin accumulation in humans, in which age pigments are regularly and heavily accumulated in cells of the inferior olivary nucleus (Mann et al., 1974). Anterior horn cells (Tomlinson & Irving, 1977) and neurones of the lateral geniculate bodies (Scholtz & Brown, 1978) have also been found to harbour particularly large amounts of lipofuscin. Among lysosomal storage diseases of humans and of domestic animals, presence of apparent topographical differences in the accumulation of storage material in the central nervous system has been reported in bovine mannosidosis by Jolly & Thompson (1978).

Regional differences in pigment accumulation may correlate more with the different cell types than with different topographical areas of the brain. Generally the amount of pigment material is much less in smaller nerve cells, such as the granular cells of the cerebellar cortex (Koppang, 1973/74). In an experiment on inducing the formation of lysosome-associated granular aggregates by injections of leupeptin, Ivy et al. (1984) revealed that large cells such as Purkinje cells and neocortical pyramidal cells of motor cortex in the brain of treated rats, were more affected with ceroid-lipofuscin accumulation than others. However, Mann & Yates (1978) reported that Purkinje cells of the cerebellum were almost free of age pigment.

To explain the occurrence of the regional differences in lipofuscin accumulation, Brizzee et al. (1974) suggest the following matters as possible sources for the effect: regional differences in energy metabolism, cell type, neurone to glia ratios, water content, type and level of enzyme activity, organelle density, as well as a host of other unique chemical and morphological features known to characterize different cortical and subcortical pathways and centres of the brain.

In ovine ceroid-lipofuscinosis, secondary changes in the brain, such as neuronal death, fibrillary astrogriosis and macrophage reaction, are present by 2.5 months of age, whereas, progressive neuronal loss is said to commence around the age of 12 to 14 months in the brain of the dog with ceroid-lipofuscinosis (Koppang, 1973/74). In both of these animal diseases, progressive neuronal loss results in marked destruction of many griseae of the brains by the age of 20 to 24 months.

Storage material is abundant in neurones early in life (Fig. 3.3b). Degeneration of neurones was noted by 2.5 months of age (see p.30) and was clearly demonstrated at 5 months of age (Fig. 3.8). Despite this, careful neurological examination failed to show evidence of a neurological defect at 6 months of age (Mayhew et al., 1985). However, by 7 months of age, affected lambs were showing neurological manifestations of the disease, these being characterized by deteriorating vision, depression, abnormal behaviour and tremor. The absence of early functional disorders despite early neuronal degeneration, is one of the common features in human degenerative neurological diseases including Alzheimer's and Pick's diseases, and indicates the presence of some compensation mechanism in the brain. For instance, Agid & Blin (1987) postulate that the

deficient neuronal transmission is compensated for by a number of physiological processes, such as hyperactivity of the remaining neurones and hypersensitivity of specific postsynaptic receptors.

Goebel et al. (1979) point out that in the human syndromes, the process which leads to the increased accumulation of lipopigment bodies damages the neurones exclusively. This observation was also noted in the study of ovine ceroid-lipofuscinosis by Janmaat (1979). However, secondary degeneration in parenchymal organs could occur and this might be masked by their regenerative ability which nervous tissue does not have. Underlying mechanisms for the secondary degeneration mentioned above are unknown. The consequences of ceroid or lipofuscin accumulation to the functioning of cells are also poorly understood (Ivy et al., 1984). Significant cell death with relatively small amounts of storage material have been revealed by longitudinal studies in human ceroid-lipofuscinosis (Zeman & Siakotos, 1973) and in canine ceroid-lipofuscinosis (Koppang, 1973/74). This contrasts with the enormous cytoplasmic distention by residual bodies without apparent evidence of cell death, which is commonly observed in classical lysosomal storage diseases such as Tay-Sachs and Niemann-Pick diseases. On this basis, Zeman & Siakotos (1973) suggested a toxic effect on the nerve cell by the lipopigment in ceroid-lipofuscinoses rather than mechanical disturbances .

This longitudinal study of ovine ceroid-lipofuscinosis has demonstrated age related differences in the topography of neuronal loss which is not recorded in previous studies of the syndromes of humans and other animals. However, these latter have usually involved terminal disease in which time-course changes are obscured. In the affected sheep, neuronal loss occurs first and is most severe in the telencephalic isocortex.

This is followed by moderately severe loss of neurones in nuclei in outer area of the hypothalamus and the anterior colliculus of the mescencephalon. Mild neuronal loss occurs later in the disease in the hippocampus, basal ganglia, thalamus and subthalamus. The cerebellum, spinal cord, pons and medulla oblongata appear to be relatively spared. In the telencephalon of affected sheep, neuronal loss occurs earlier and is more severe in the parietal lobe. This is followed by less severe loss of neurones in the occipital and frontal lobes. Mild neuronal loss occurs in the temporal lobe later in the disease. In the telencephalic isocortex, neuronal loss occurs first and is most severe in the middle area. It gradually extends into other areas until terminal disease. On the other hand, in the brain of dog with ceroid-lipofuscinosis, nerve cell loss was recorded throughout the grey matter of the brain and it was most severe in the cerebellar cortex (Koppang, 1973/74). Presence of topographical differences in neuronal death between the different brain lobes leading to gross atrophy of the particular brain lobes have been reported in the human degenerative neurological diseases such as Alzheimer's disease (Terry et al., 1981) and Pick's disease (Tomlinson & Corsellis, 1984). In both of the diseases, frontal and temporal lobes are selectively affected.

There are several reports which describe the presence of selective neuronal loss of certain cell types in the brain of the patients with ceroid-lipofuscinosis. The cell type affected differs between the reports. Significant loss of small nerve cells such as the granule cells of the cerebellar cortex (Zeman & Siakotos, 1973; Koppang, 1973/74) and the olfactory bulb (Zeman & Siakotos, 1973) are reported in the late infantile form of the human syndrome and in the canine syndrome. Braak & Goebel (1978, 1979) noted that certain types of neurones were selectively affected with degenerative changes in the

telencephalic isocortex of patients with juvenile form of the syndrome. Similar findings were also observed in familial protracted juvenile form and adult form of the disease (Goebel et al., 1982b). In these brains, loss of pigment-laden stellate cells in the outer cellular laminae and of layer Va-pyramidal cells, and enlarged initial axonal segments of layer IIIab-pyramidal cells were prominent. These changes are considered to be specific to the ceroid-lipofuscinoses (Goebel et al., 1982a). Braak & Goebel (1978) postulated that the selective involvement of the small pigment-laden stellate cells, which are known as local circuit neurones, may occur first due to their small size and early onset of lipopigment accumulation. This may be followed by changes in layer IIIab- and layer Va-pyramidal cells which are probably associated with the pigment-laden stellate cells by interlaminar connections (Braak & Goebel, 1979). Occurrence of selective neuronal loss in certain areas of the cerebral isocortex was also noted in the present study of ovine ceroid-lipofuscinosis, being associated with a laminar destruction of the isocortex. However, no particular type of neurone affected was identified by the methods used. Similar changes may also take place in human degenerative neurological diseases. Vogt (1928) and Sciffer (1955) contended that in Pick's disease there was a sequence to the cell loss, layer III being the first to degenerate followed by layers II and IV. Prominent neuronal loss in the third layer was also described in Huntington's chorea by Alzheimer (1911).

During the destruction of the telencephalic isocortex in ovine ceroid-lipofuscinosis, focal arrangements of neurones were seen in the superficial area with their nuclei being abnormally close together and in which glial nodules were occasionally noted (Figs. 3.8, 3.9 & 3.10). This may reflect a degenerative change in the neurones.

In mammals, most of projection fibres, which give rise to the corticospinal system, arise from large motor neurones in the deeper layers of the cortex, while the association and commissural fibres, which interconnect various cortical regions, arise mainly from more superficial small neurones (Jones & Wise, 1977). In ovine ceroid-lipofuscinosis, even in severely destroyed cerebral cortex of terminally affected sheep, some pigment laden large pyramidal neurones may remain. These factors may explain why Wallerian type degeneration and gliosis were most severe in the subcortical white matter while they were mild in the lower regions of the corticospinal system of affected sheep (Table 3.IV).

An severe astrocytosis is one of the striking features of the ceroid-lipofuscinoses. It appears due not only to hyperplasia of astrocytes but also to condensation of brain tissue associated with severe neuronal loss. Astrocytes are also hypertrophied and may have bizarre shaped nuclei. It is not clear whether astrocytosis merely reflects neuronal degeneration and loss or a toxic insult inherent in the lipopigment or the altered metabolism of cells leading to pigmentation.

CHAPTER IV

ELECTRON MICROSCOPY

I. INTRODUCTION

Electronmicroscopically the cytoplasmic pigment granules are seen to be irregularly rounded electron dense bodies. They are bound by a trilaminar membrane, and are composed of a granular matrix and lamellar profiles described as "curvilinear" and "fingerprint" patterns (Zeman & Siakotos, 1973).

The fine structure of the pigment granules reported in animals with analogous disease is essentially the same as that of humans. In a longitudinal study on English Setter dogs with ceroid-lipofuscinosis, Koppang (1973/74) revealed that the pigment was already present at birth.

This chapter reports a longitudinal study on the pathogenesis and ultrastructure of pigment granules in sheep affected with ceroid-lipofuscinosis.

II. MATERIALS AND METHODS

Animals

Animals used in this study are described in Tables 2.I and 2.II.

Preparation of tissues for electron microscopy

For transmission electron microscopy, fresh cerebral cortical tissues were obtained shortly after death from dystocia (control 1), immediately after caesarean section (experimental cases 2 and 3) or euthanasia (experimental cases

5-11,15,16 and controls 3-8). They were trimmed into 1 mm^3 cubes which were fixed in 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 3 h. Primary fixed tissues were washed in phosphate buffer (x3) and post fixed in 1% osmium tetroxide in phosphate buffer (pH 7.2) for 1 h. They were then washed in buffer, put through a graded series of ethanol and propylene oxide and embedded in epoxy resin (Durcupam-ACM, Fluka, Switzerland). For light microscopy thin sections 0.5 to 1.0 µm in thickness were cut on a LKB III ultramicrotome (LKB-Produkter AB S-161 25 Bromma 1, Sweden). Ultrathin sections for electron microscopy were cut at 70 nm and mounted on unsupported copper grids. These sections were stained in 50% ethanol with saturated uranylacetate for 6 min, and in lead citrate for 6 min and examined in a Philips 200 electron microscope. For the mid term (experimental case 1) and full term (experimental case 4) foetuses, formalin fixed brains were trimmed into 1 mm^3 cubes, post fixed in osmium tetroxide and then treated as above.

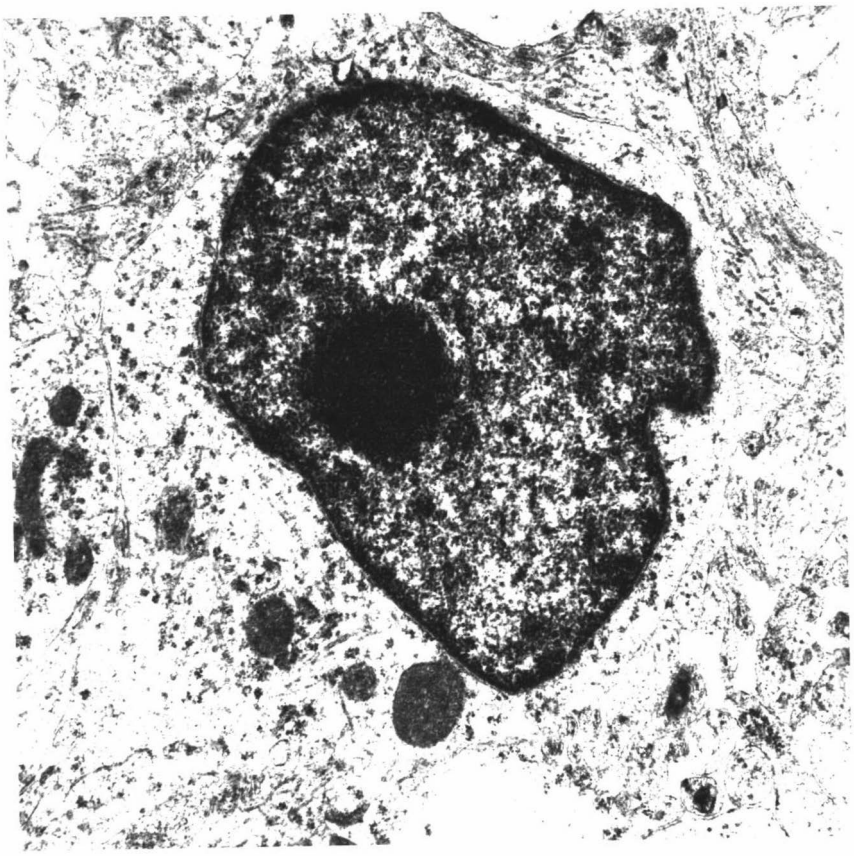
III. RESULTS

Electron microscopy of brain of affected sheep

Irregularly rounded electron dense bodies 0.2 to 15 µm in diameter are present in neuronal perikarya in brains of all sheep affected with ceroid-lipofuscinosis, including mid term foetuses (Fig. 4.1). The size and number of these pigment cytosomes increase with age; the larger appear to be formed by coalescence of the smaller dense ones (Fig. 4.2). These electron dense bodies are also noted in axons (Fig. 4.3), astrocytes (Fig. 4.4), macrophages (Fig. 4.5) and endothelial cells of blood vessels (Fig. 4.6). These become more obvious with age.

Figure 4.1: Cerebral cortical neurone from a mid term ovine foetus affected with ceroid-lipofuscinosis. Note presence of electron dense residual bodies 0.7 µm in diameter in the cytoplasm. (EM x17,700)

Figure 4.2: Part of a cerebral cortical neurone of a 7 months old affected lamb. This cytosome appears to be formed by coalescence of smaller dense bodies. (EM x38,000)



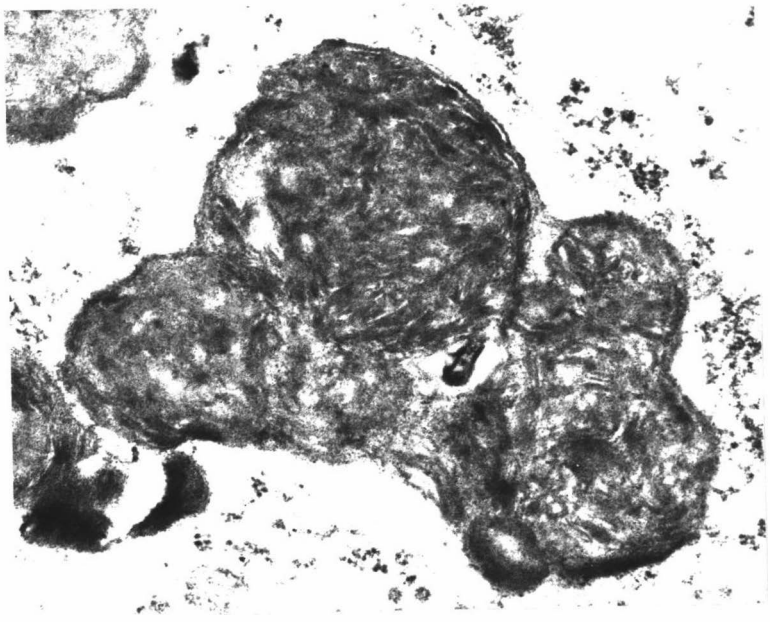
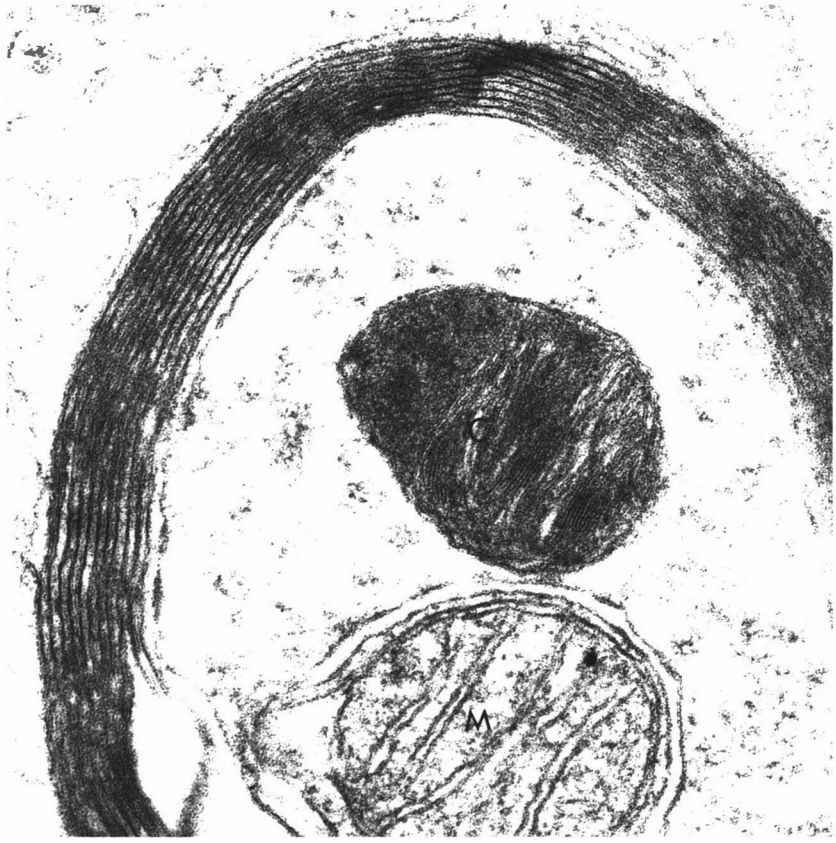


Figure 4.3: Myelinated axon in the cerebral cortex of a 22 months old affected sheep contains a storage cytosome (C) and a mitochondria (M). (EM x97,200)

Figure 4.4: Astrocyte (A) in the cerebral cortex of a 7 months old affected lamb contains storage cytosomes and thick bundles of fibrils in a hypertrophic process. It is adjacent to the neurone (N) that is interpreted as showing signs of degeneration. (EM x6,000)


C
M

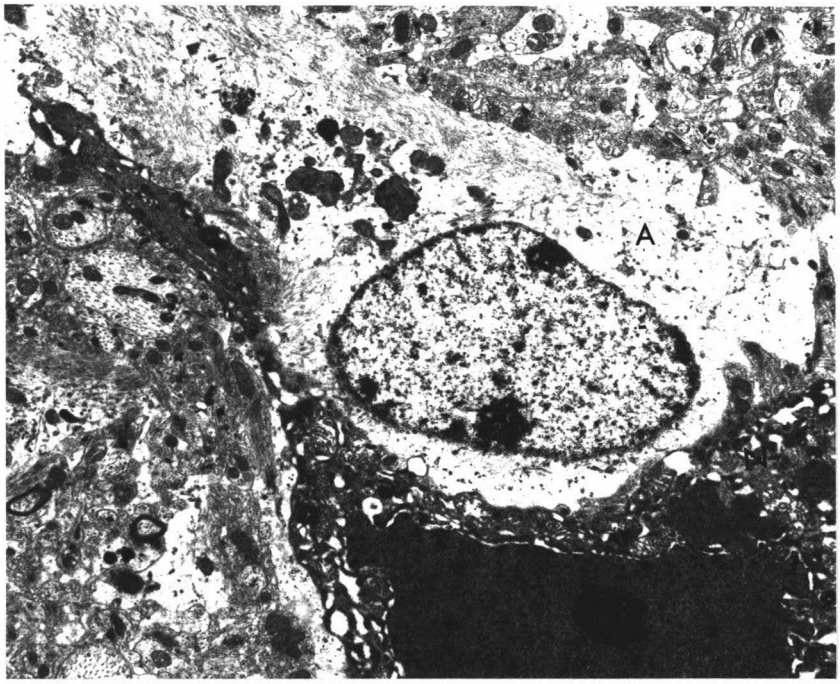
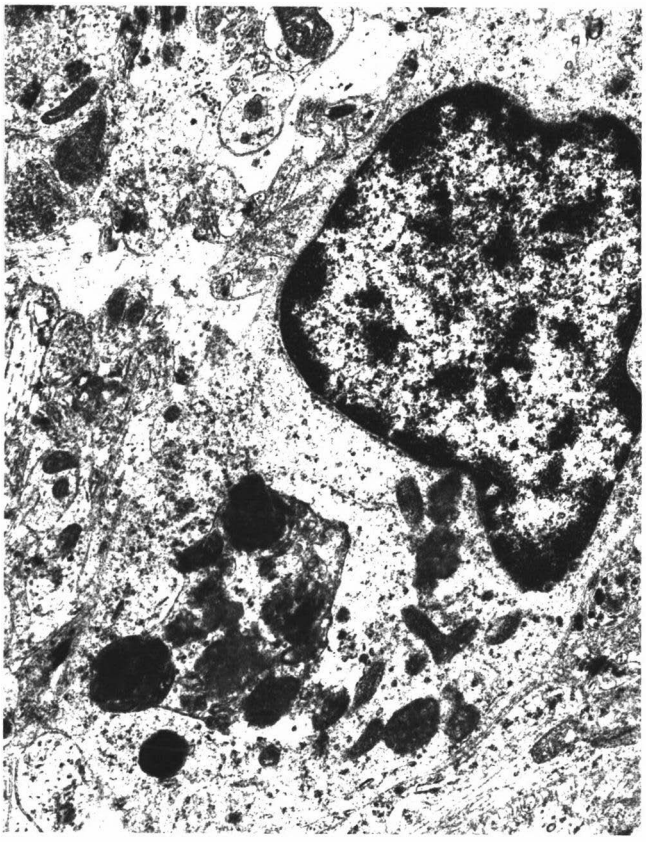
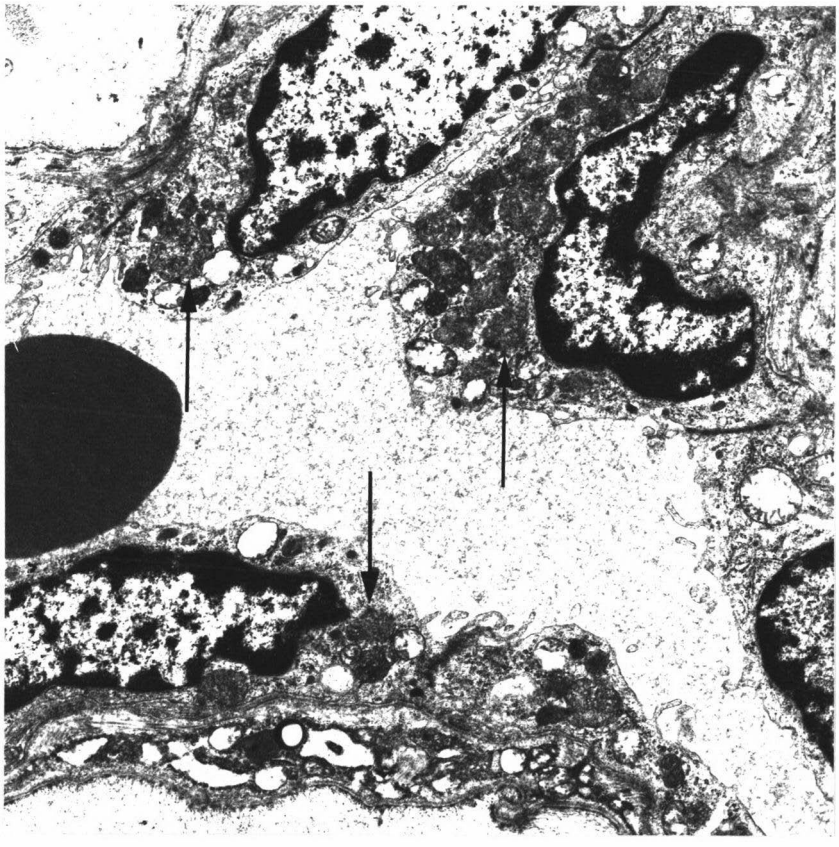

A
P

Figure 4.5: Discrete residual bodies within a membrane bounded cytosome in a macrophage in the cerebral cortex of a 7 months old affected lamb. (EM x13,000)

Figure 4.6: Blood vessel in the cerebral cortex of a 22 months old affected sheep. Note electron dense bodies in the endothelial cells (arrows). (EM x98,000)



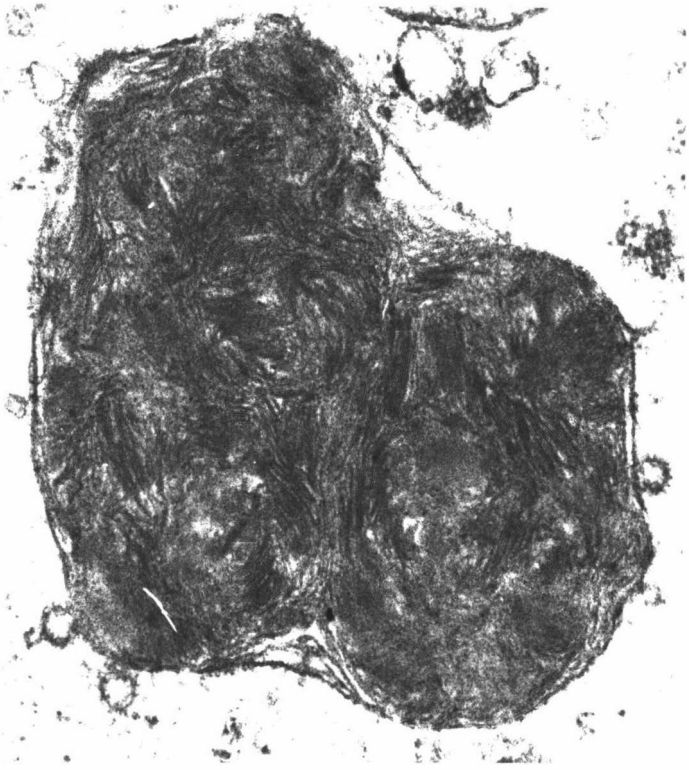


The pigment cytosomes are often seen to be bounded by a discrete trilaminar membrane (i.e. typical lipid bilayer membrane) (Fig. 4.7). This is particularly well seen in the smaller bodies less than 2 µm in diameter. Their internal structure appears to be composed of a granular matrix but includes a variety of multilamellar profiles similar to those described in the literature on the human syndromes. These have been named curvilinear (Fig. 4.8), fingerprint (Fig. 4.8), tubular (Fig. 4.9), crystalloid (Fig. 4.10) and multilamellar (Fig. 4.11 & 4.12). At a high power magnification, many of these lamellar arrays appear to consist of alternating electron dense and less dense lines being separated by translucent layers. These may be as simple as three-layered (Fig. 4.11) or five-layered (Fig. 4.12) lamellae. The thickness of the former lamellae are approximately 6.5 nm, this appears to be similar to that of the surrounding membrane of the pigment cytosomes. The latter lamellae which measure about 12 nm in thickness are composed of a major denser and thicker middle line flanked on either side by translucent layers and two electron dense outer lines. Electron-lucent vacuoles (Fig. 4.9) 0.5 to 1.0 µm in diameter are very rarely contained in this storage material.

Although several pigment cytosomes as described above are present in neurones of foetal brains, the majority of abnormal neuronal pigment cytosomes in full term foetal brain are much smaller and less complex (Fig. 4.13). Occasional less complex smaller cytosomes are also noted in mid term foetal brain. These measure 0.2 to 1.5 µm in diameter and are clearly defined by a discrete trilaminar membrane (Fig. 4.14). They are located diffusely in the cytoplasm of neurones and occasionally in neurites (Fig. 4.15). The fine structure of these smaller cytosomes is composed of either coarse or fine granular matrices and a three-layered membranes which are similar to those of the limiting membranes (Fig. 4.16). These are present

Figure 4.7: Electron dense body within a cerebral cortical neurone from a full term ovine foetus with ceroid-lipofuscinosis. Note a surrounding trilaminar membrane (i.e. lipid bilayer membrane). (EM x51,200)

Figure 4.8: Part of a storage cytosome within a cerebral cortical neurone from an affected full term ovine foetus, showing fingerprint and curvilinear profiles. (EM x83,500)



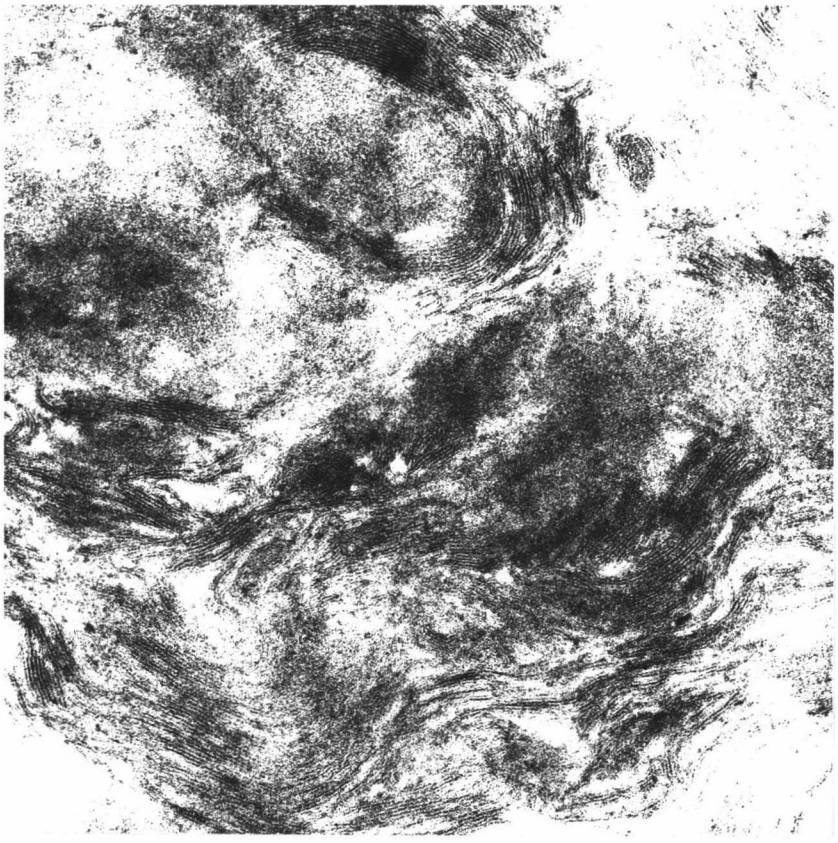
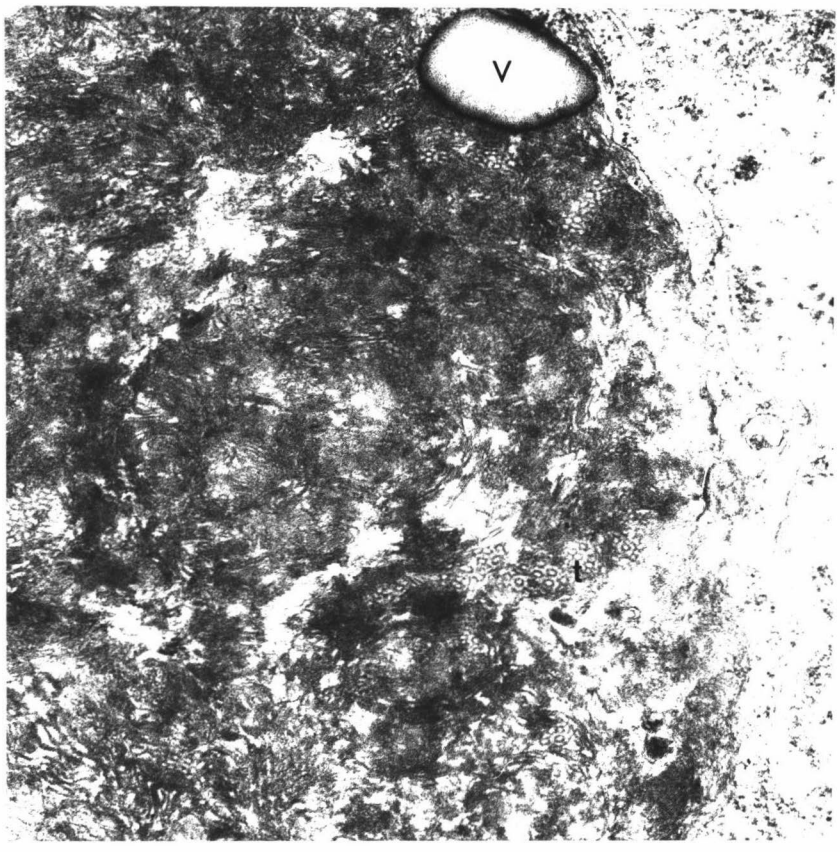


Figure 4.9: Storage cytosome within a cerebral cortical neurone from a 22 months old affected sheep, showing a "tubular" structures (t) and an electron-lucent vacuole (V). (EM x36,800)

Figure 4.10: Part of a storage cytosome within an astrocyte in the cerebral cortex of a 22 months old affected sheep, showing a crystalloid (c) pattern. Note glial fibrils (F) on the left. (EM x120,000)


V

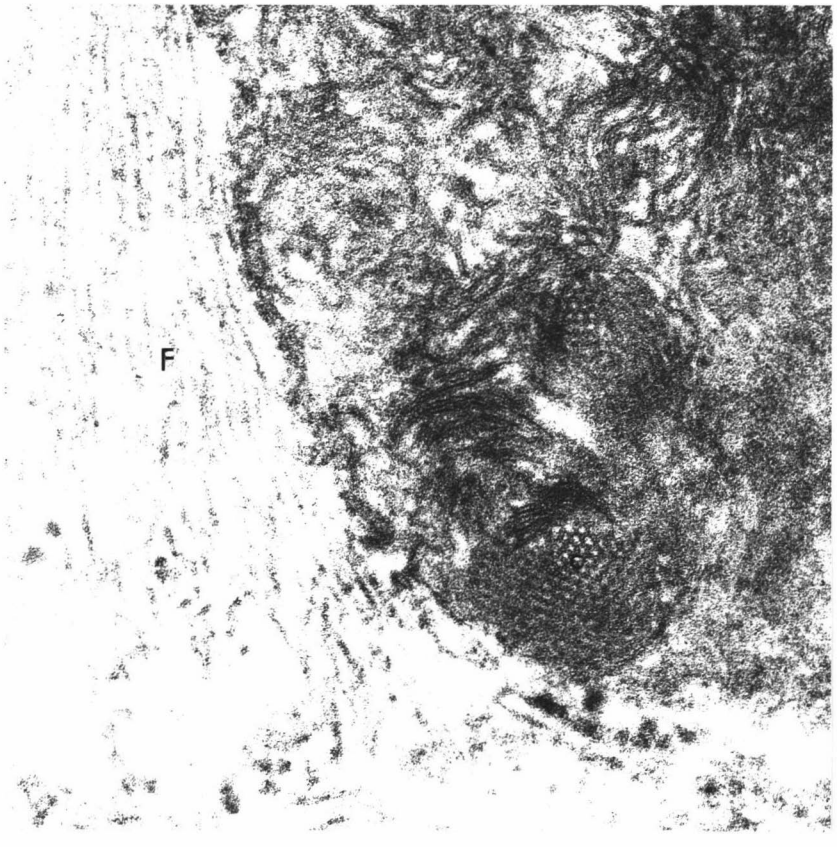
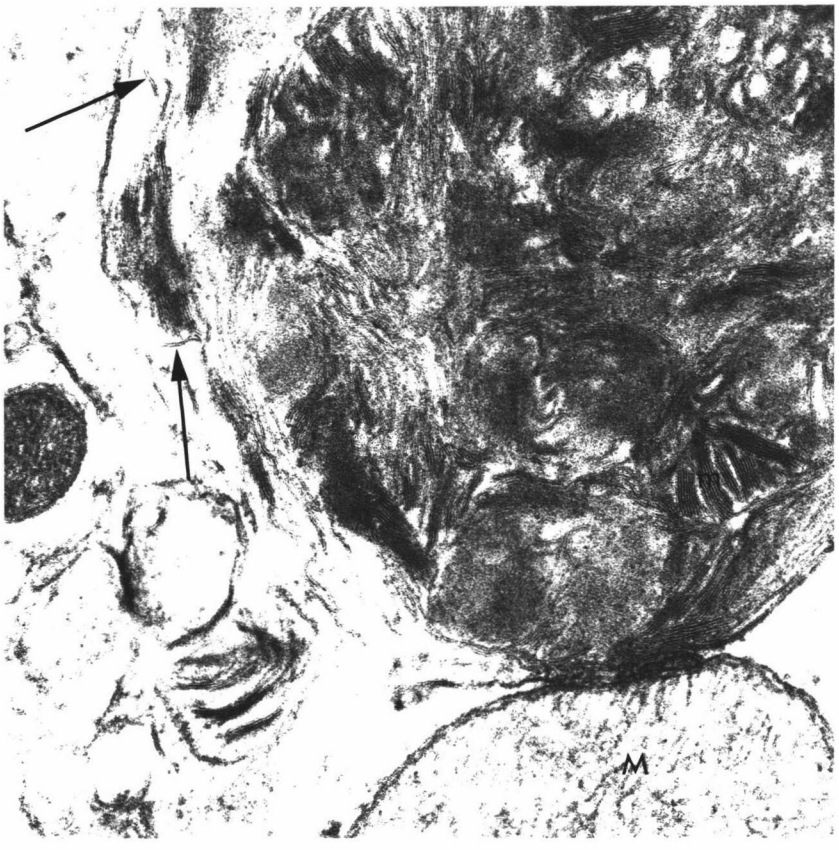

F

Figure 4.11: Part of a cerebral cortical neurone from a full term ovine foetus with ceroid-lipofuscinosis. Note electron dense body, showing three-layered lamellae (arrows) with approximately 6.5 nm in thickness and stacks of multilamellar arrays (m). On the bottom is a mitochondria (M). (EM x71,600)

Figure 4.12: Part of a multilamellar cytosome within a cerebral cortical neurone from a full term ovine foetus with ceroid-lipofuscinosis. It shows five-layered lamellar structures (arrows) with a periodicity of approximately 12 nm and stacks of multilamellar arrays. (EM x91,000)


M

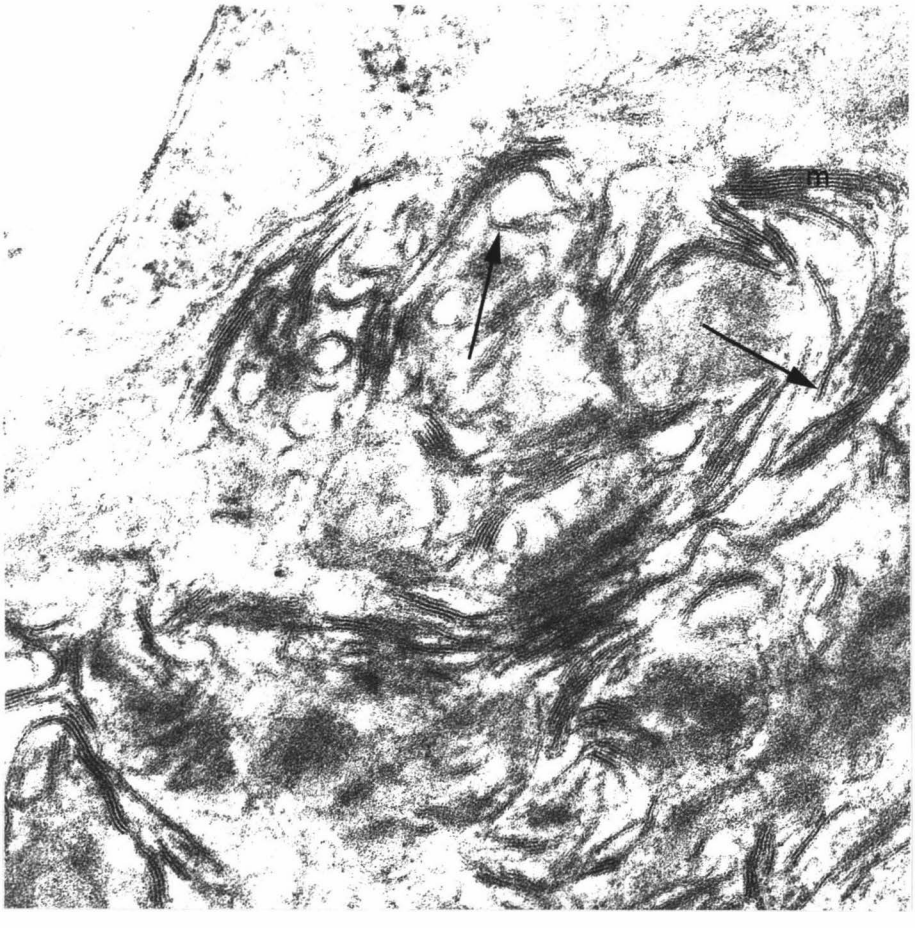

m

Figure 4.13: Part of a cerebral cortical neurone from a full term ovine foetus with ceroid-lipofuscinosis, showing a variety of abnormal pigment cytosomes less than 1.2 µm in diameter in the cytoplasm. These contrast with and are generally smaller than mitochondria. (EM x14,000)

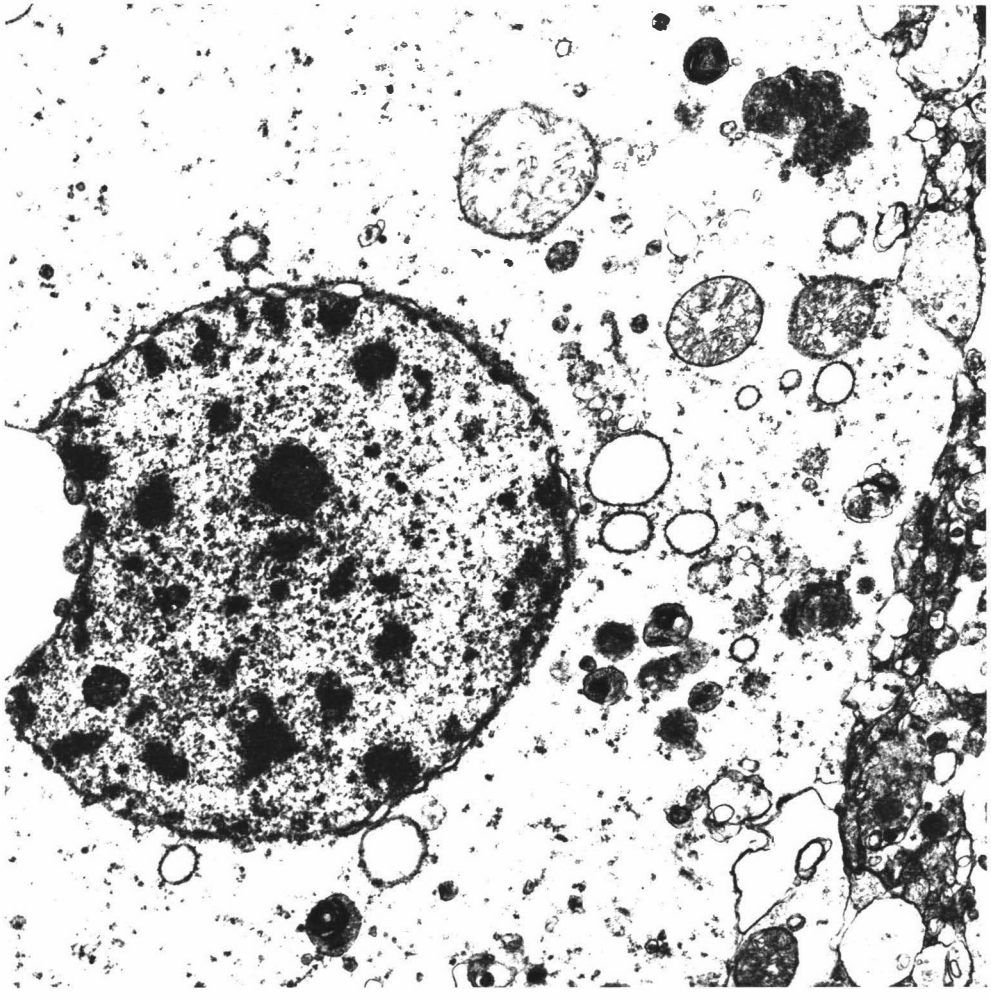
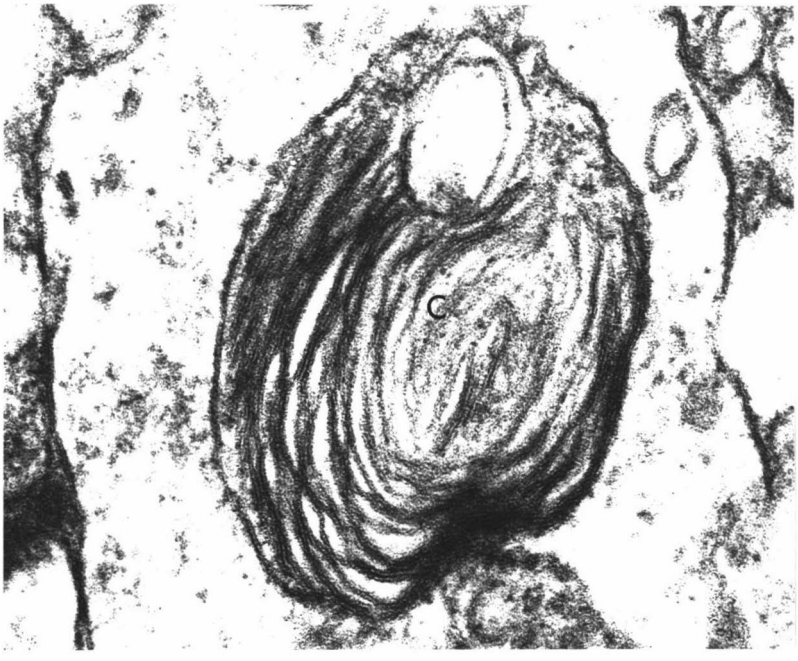


Figure 4.14: Pigment cytosomes up to 0.8 µm in diameter within a cerebral cortical neurone from a full term ovine foetus with ceroid-lipofuscinosis, showing a discrete surrounding membrane. Note a granular matrix and multilamellar components within these cytosomes. (EM x71,600)

Figure 4.15: Part of a neurite in the cerebral cortex of a full term ovine foetus with ceroid-lipofuscinosis, showing a multilamellar cytosome (C). (EM x106,200)


C

as whorls or loose stacks (Fig. 16). In some electronmicrographs, there is a suggestion of continuity between the surrounding membrane and the internal membranes (Figs. 4.16 & 4.17). Some of the small cytosomes also contain multilamellar structures (Fig. 4.18). Lamellar arrays changing to a fine granular matrix are frequently observed (Fig. 4.19).

The fine structure of dense bodies in macrophages varies considerably. They include pigment cytosomes of similar structure to those in neurones, amorphous electron dense material, membranous bodies and degenerated myelin (Fig. 4.20).

Degenerating myelinated axons are occasionally noted in electron micrographs of the cerebral cortex. Hypertrophic fibrillary astrocytes with bizarre nuclei and a dense network of fibrous processes are a dominant feature in the latter stages of the disease (Fig. 4.21).

Electron microscopy of brain of normal sheep

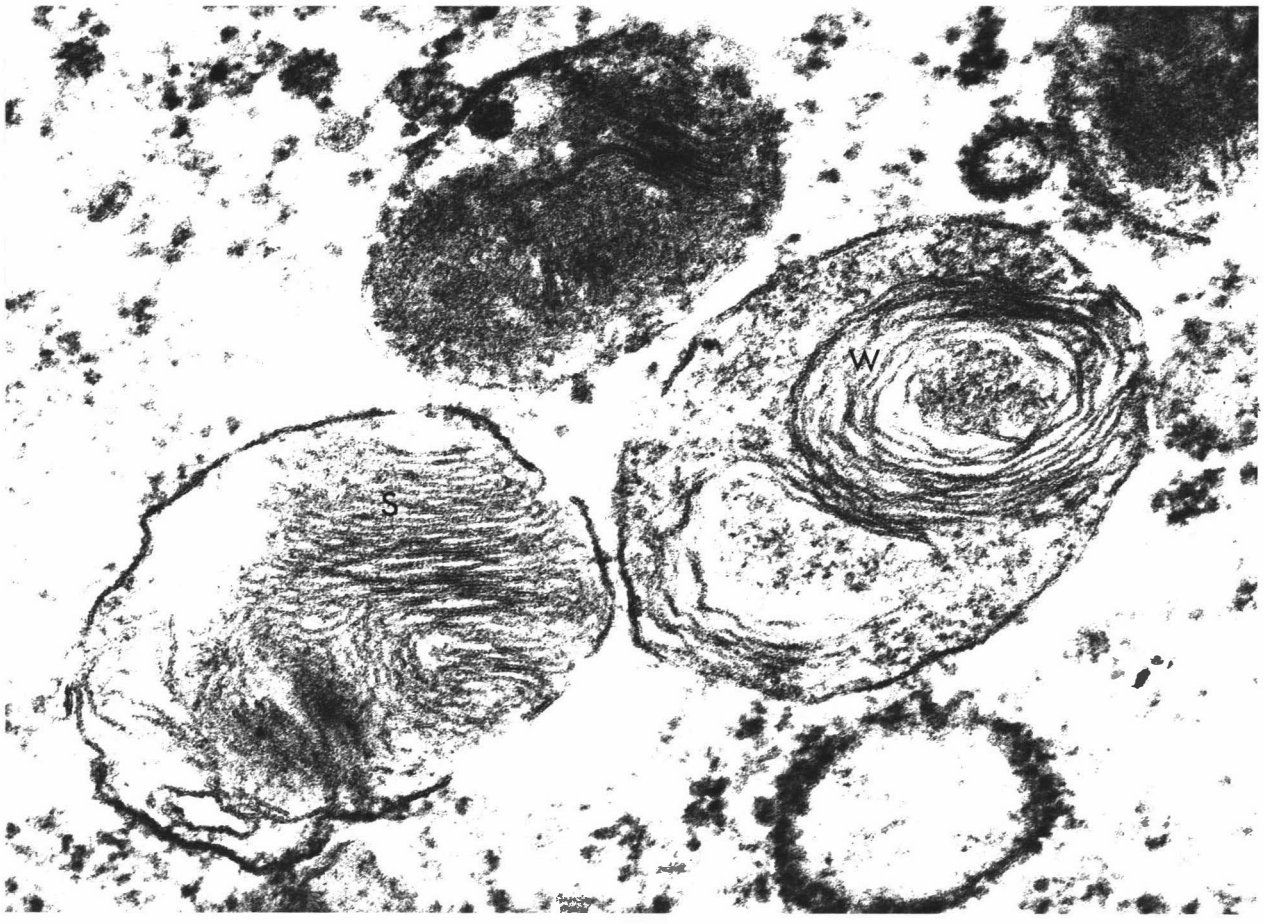
The cytoplasmic fine granules noted by light microscopy in neurones in brains of normal sheep are membrane-bound electron dense bodies 0.5 to 1.0 µm in diameter, in which a fine granular matrix and occasional electron-lucent vacuoles 0.3 to 0.5 µm in diameter are noted (Fig. 4.22). These structures are found in neurones of both full term foetal and mature brains and are clearly different from the disease related cytosomes.

IV. DISCUSSION

The salient ultrastructural findings noted in the present study of ovine ceroid-lipofuscinosis were the detection of complex electron dense cytosomes and less complex cytosomes of smaller size, in mid term (Fig. 4.1) and full term (Fig. 4.13)

Figure 4.16: Part of a cerebral cortical neurone from a full term ovine foetus with ceroid-lipofuscinosis, showing lamellar cytosomes less than 0.8 µm in diameter. Note whorls (W) and loose stacks (S) of trilaminar membranes. (EM x102,300)

Figure 4.17: Higher power magnification of one part of the lamellar cytosome with whorls in Fig. 4.16. Arrow points to the site at which continuity between the surrounding membrane and the internal membrane is suggested. (EM x153,000)


W
S

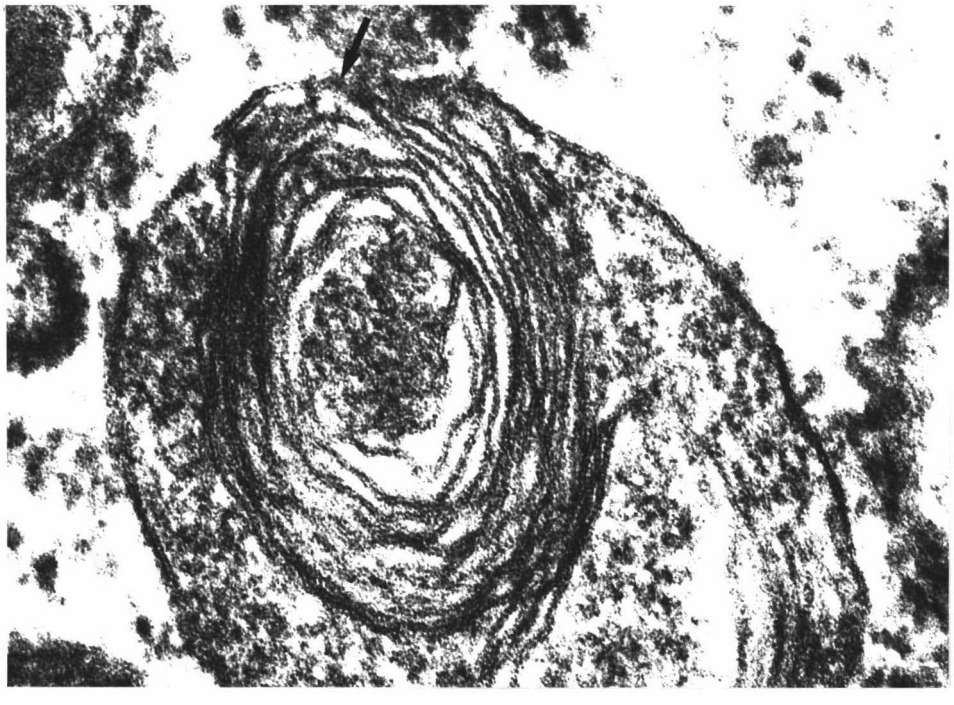
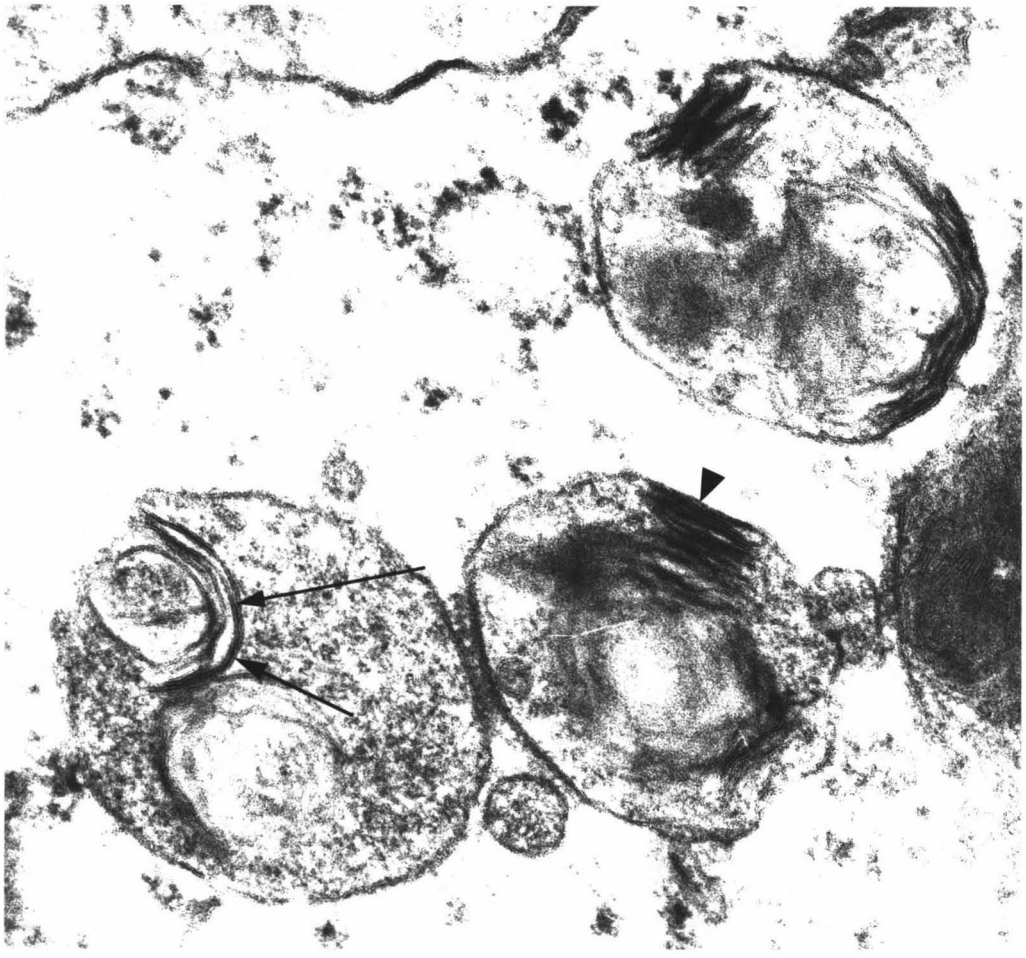


Figure 4.18: Part of a cerebral cortical neurone from a full term ovine foetus with ceroid-lipofuscinosis, showing a variety of storage cytosomes. Multi-layered (arrow head) and five-layered lamellae (short arrow) are seen in the cytosomes. The long arrow indicates the site at which a five-layered lamella appears to be formed by the fusion of two three-layered lamellae. (EM x87,000)

Figure 4.19: Part of a cerebral cortical neurone from a full term ovine foetus with ceroid-lipofuscinosis, showing a cytosome (C) on the lower right, in which lamellar arrays appear to change to a fine granular profile (arrow). Note multivesicular body (B), swollen rough endoplasmic reticulum (R) and part of a mitochondria (M). (EM x76,700)



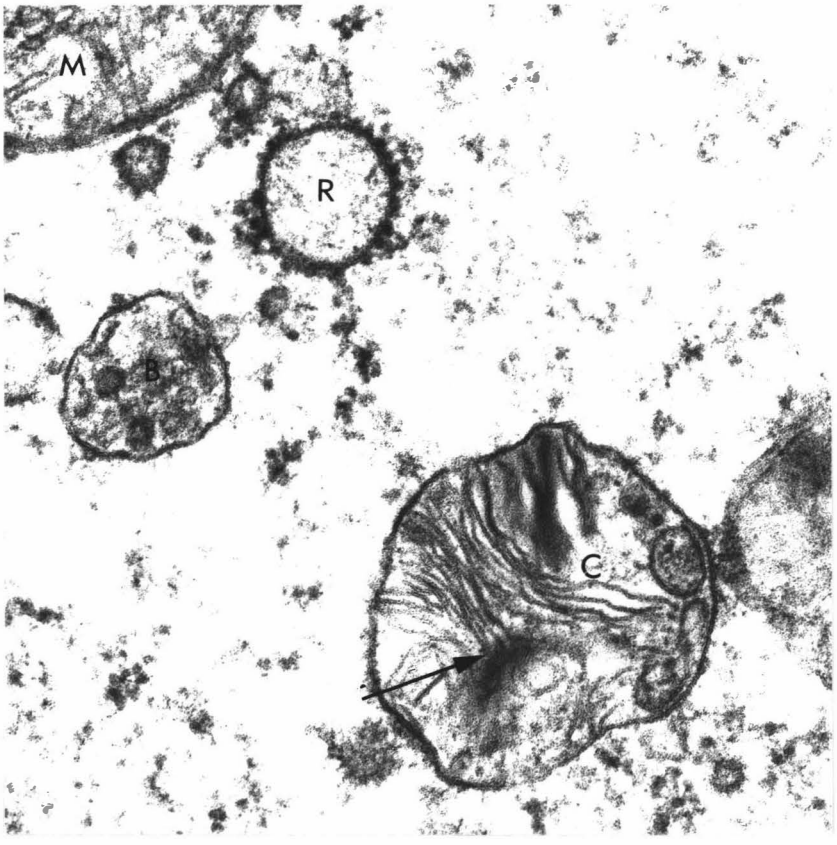

M
R
B
C

Figure 4.20: Macrophage in the cerebral cortex from a 9 months old affected lamb, containing storage cytosomes of similar structure to those in neurones (C), amorphous electron dense material (m), membranous bodies (B) and degenerated myelin (M) in the cytoplasm. (EM x73,600)

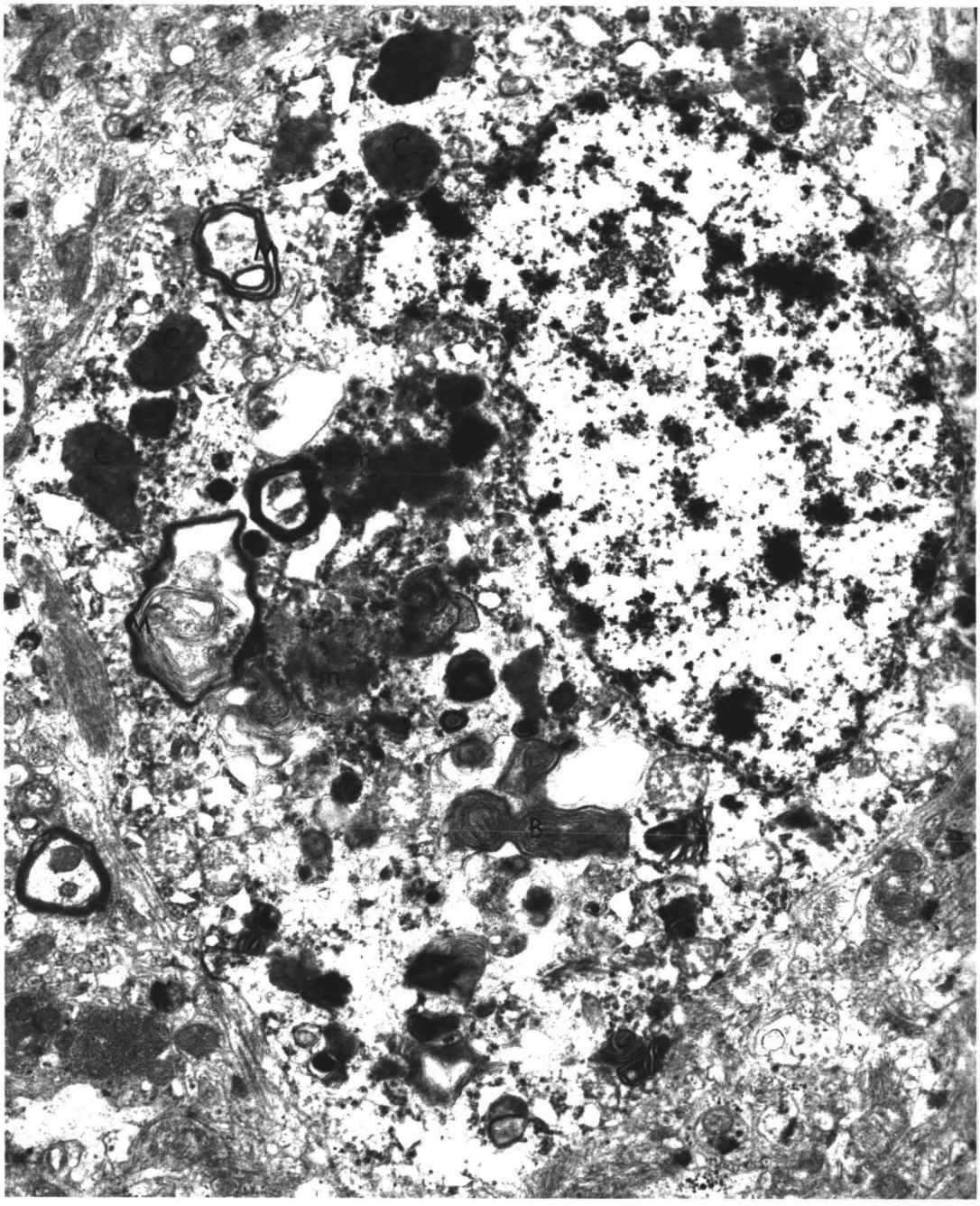
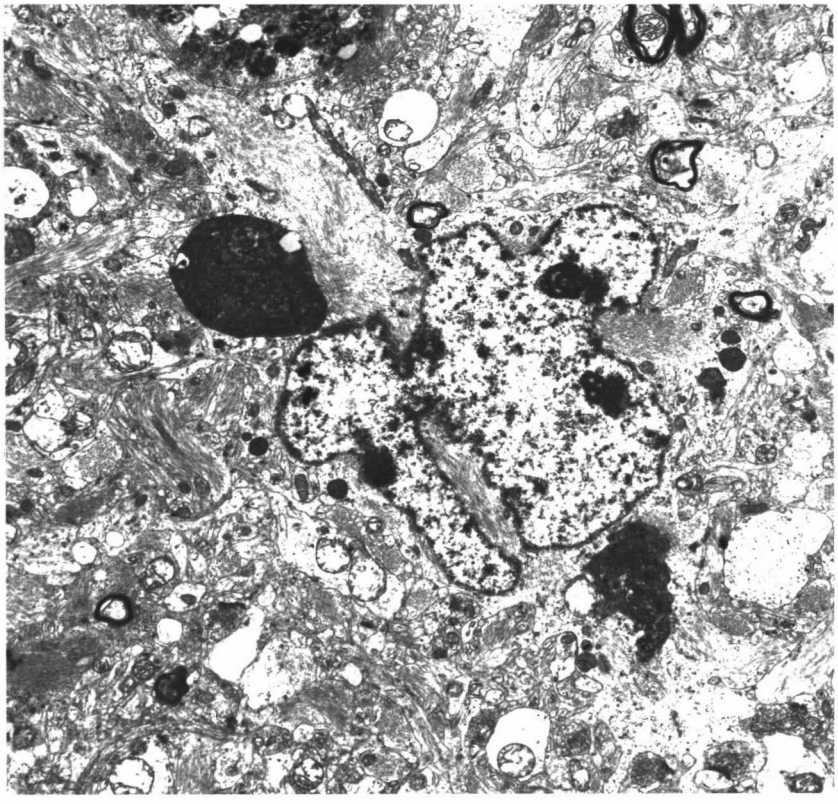
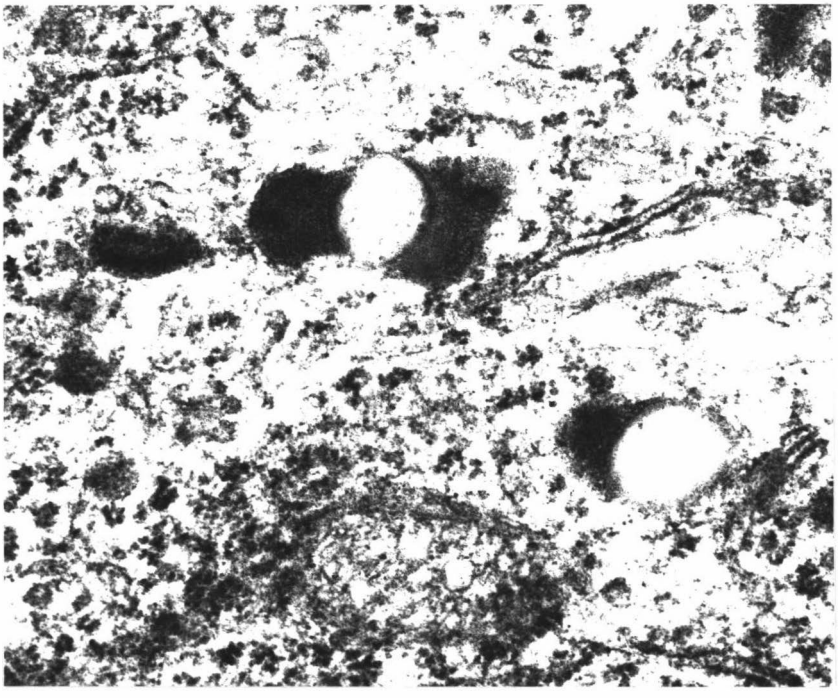

C
C
C
M
M
B

Figure 4.21: Astrocyte in the cerebral cortex from a 22 months old affected sheep showing a nucleus of bizarre shape and a hypertrophic process with increased number of fibrils and storage cytosomes. Note dense network of astrocytic fibrous processes in the adjacent neuropile. (EM x6,000)

Figure 4.22: Part of a cerebral cortical neurone from a normal full term ovine foetus, showing residual bodies composed of a fine granular matrix and an electron-lucent vacuole. (EM x53,600)





affected foetal brains. This confirms the presence of Sudanophilic lipopigment granules in affected foetal brains noted by light microscopy. Thus, it is concluded that formation of autofluorescent cytosomes commences *in utero*. Koppang (1973/74) also detected typical membrane-bounded ceroid bodies by electron microscopy in the neurones of the spinal cord of 2-day-old affected puppy and concluded likewise.

In ovine ceroid-lipofuscinosis, the pigment cytosomes, particularly the less complex ones were frequently defined by a trilaminar membrane (Fig. 14). This probably reflects the lysosomal nature of the cytosomes. A conclusion was also supported by their lipid composition (Palmer *et al*. 1986b). Some larger electron dense cytosomes appeared to be formed by coalescence of smaller complex cytosomes. Typical complex electron dense cytosomes were composed of a granular matrix and a variety of multilamellar profiles, such as curvilinear (Fig. 4.8), fingerprint (Fig. 4.8), tubular (Fig. 4.9), crystalloid (Fig. 4.10) and multilamellar (Fig. 4.11 & 4.12) which are similar to those described in analogous human diseases.

Lipopigment cytosomes isolated from affected sheep contain a high proportion of protein (approximately 70%) (Palmer *et al*., 1986a, 1986b). Much of this was composed of two specific peptides of 14,800 and 3,500 M_r which appeared specific for the disease. On the basis of these findings and similar complex lamellar structures given in the poly-L-lysine/cardiolipin multilaminar complex (De Kruijff *et al*., 1985) and in the myelin sheath of nerves (Rumsby, 1978), Jolly *et al*. (1987) suggested that multilamellar structures of the lipopigment cytosomes in affected sheep may have a complex three dimensional structure. It may be like the "Dannielli and Davson" (1935) type structure but with a number of alternate layers of lipid sandwiched between and interacting with layers

of hydrophobic protein. This hypothesis was supported by the results of powder x-ray diffraction study in which there were two broad peaks with diffraction spacings between 9.8 and 10 Å and 4.5 and 4.6 Å. It was postulated that the specifically stored peptides were repeating unit that dictated the structure. Since the word "membrane" implies either a lipid bilayer membrane or a simple planar structure, Jolly et al. (1987) proposed to use the term "lamellar" in order to describe the components of the complex three dimensional structure as seen by electron microscopy. It must be remembered that ultrastructural appearance may reflect modifications of the true structure induced by fixation, dehydration and embedding processes.

At a high power magnification, some lamellar profiles appear to consist of three- or five-layered lamellae (Figs. 4.11 & 4.12). In canine ceroid-lipofuscinosis, Koppang (1973/74) proposed that the membranous stacks were derived from fusion of adjacent less dense outer lines of original five-layered - "membranes". However, tubular and crystalloid patterns appear to occur as a result of intersecting lamellae. Coarse granular profiles observed diffusely in the granular matrix may also, in part, be due to cross sectioning of the lamellar structures. Much of the granular matrix is also likely to be lamellar but with the lamellae not sectioned in such a plane as to show their true structures (Jolly et al., 1987).

Some of the small cytosomes contained whorls or loose stacks of trilaminar membranes similar to those of the limiting membranes (Fig. 4.16). In some electronmicrographs, there was a suggestion of continuity between the surrounding membrane and the trilaminar membrane (Figs. 4.16 & 4.17) but this was not definitely demonstrated. This is provisionally interpreted as being due to an internalization of surrounding limiting

membrane rather than a recycling of membrane. Some of these small cytosomes also showed some multilamellar profiles (Fig. 4.18) suggesting that the latter resulted from condensation and alteration of the more simple membranous structure. As the less complex smaller cytosomes were noted in affected foetal brains, it is probable that they are an early stage of the more complex ones. Presence of immature storage cytosomes in human foetal brains has also been reported in lysosomal storage diseases such as Tay-Sachs disease (Adachi et al., 1974), mucopolysaccharidosis II (Lake, 1984), and Niemann-Pick disease (Schneider et al., 1972).

The electron dense bodies observed in brains of normal control sheep, including a full term foetus, were surrounded by a membrane and were composed of a fine granular matrix and occasional electron-lucent vacuoles (Fig. 4.22). This internal structure appears to be typical of lipofuscin (age pigment) (Siakotos et al., 1970) and is different from that of the electron dense cytosomes so commonly observed in the brain of sheep with ceroid-lipofuscinosis. Thus, electron microscopy confirms the presence of lipofuscin in the full term foetal brain as noted by light microscopy. In human nervous tissue lipofuscin has been noted in foetal spinal cord neurones (Humphrey, 1944) and in the inferior olive at 3 months (Brody, 1960). In an extensive study of nonhuman primate, Macaca mulatta, Brizzee et al. (1974) found that pigment first appeared in the inferior olive at the age of 3 months.

A dense network of fibrous processes in the terminally diseased brain (Fig. 4.21) is in accord with the intensive fibrillary astrogliosis noted by light microscopy. Macrophages, apart from typical multilamellar cytosomes also contained more amorphous electron dense material and myelin debris (Fig. 4.20). This heterogeneity of material is also in accord with

variable staining of storage material in macrophages with lipid stains. Whereas it can be concluded that much of this material is phagocytosed and reflects degeneration of neurones and axonal sheaths, it is not clear how much is formed de novo within the macrophage as a result of the metabolic anomaly.

CHAPTER V

GENERAL DISCUSSION

A longitudinal morphological study on the central nervous system of sheep with ceroid-lipofuscinosis and of age matched controls revealed that the ovine disease has not only many neuropathological findings in common with analogous human diseases, but also some pathological features which have not been reported in affected humans or animals.

In ovine ceroid-lipofuscinosis, gross pathological changes in common with the human syndromes include striking brain atrophy, thickening of the skull bones overlying the brain and slight enlargement of the lateral ventricles. Cerebellar atrophy, which is common in the infantile and late infantile types of human syndrome (Zeman, 1976) and in the canine syndrome (Koppang, 1973/74), is not a particular feature in the ovine disease.

Histologically, ovine ceroid-lipofuscinosis, like its human and canine counterparts, is characterized by the progressive accumulation of autofluorescent lipopigment in neurones and a wide variety of other cell types (Jolly et al., 1980, 1982). In the present study of ovine ceroid-lipofuscinosis, these lipopigment granules were detected by light microscopy as early as the mid stage of foetal development, the earliest stage examined. This was confirmed by electron microscopy. Coronal sections of the whole brain of affected sheep showed that there were regional differences in the quantity of accumulated lipopigments in neurones of various areas. Similarly there were age related topographical differences in secondary degenerative changes (see Chapter III). In ovine ceroid-lipofuscinosis, the

cerebellum appears to be relatively spared these secondary changes. In contrast, neuronal loss in the cerebellar cortex is common in the human syndromes (Zeman, 1976) and in the canine syndrome (Koppang, 1973/74).

Neuronal loss in the telencephalic isocortex of affected sheep showed an initial laminar distribution (Fig. 3.8). This pattern was well demonstrated by a concomitant astrocytosis (Fig. 3.11). The laminar distribution of neuronal loss probably reflects the selective degeneration of certain types of neurone. A similar laminar distribution of neuronal loss has also been reported in the human syndromes (Braak & Goebel, 1978, 1979; Goebel et al., 1982a, 1982b). Neuronal loss in the cerebral cortex of the parietal lobe of brain of terminally affected sheep was as severe as that in the cerebral cortex described in the infantile type of human syndrome (Haltia et al., 1973a; Zeman, 1976).

Many papers have been published concerning biochemical aspects of the ceroid-lipofuscinoses. However, the underlying metabolic anomaly of the disease has not been fully elucidated. Inconsistency and variation of the biochemical results may be, in part, due to secondary changes attributable to brain necrosis and time. As shown in the electron micrograph Fig. 4.20, macrophages take up lipopigments as well as degenerating myelin. This may confound the composition of the disease specific lipopigments. Lipid staining characteristics, yellowish colouration and fluorescent spectra of the stored lipopigment suggested firstly a lipidosis and then an abnormal peroxidation of lipids (Zeman & Dyken, 1969; Zeman, 1976). The latter hypothesis gained support when Armstrong et al. (1974) reported a peroxidase deficiency in leukocytes and several tissues from patients with ceroid-lipofuscinosis. However, others (Den Tandt & Martin, 1978; Marklund et al., 1981)

reported no deficiency of such an enzyme activity. The lack of consistency in the results suggested that such a peroxidase deficiency was not the primary defect in the ceroid-lipofuscinoses (Paton & Poulos, 1984). Disturbances in polyunsaturated fatty acid metabolism have also been reported (Hagberg et al., 1968; Svennerholm et al., 1975). Based on the analysis of isolated lipopigment, alternate hypotheses have been proposed. Wolfe et al. (1977, 1981, 1983, 1987) reported an elevation in the level of dolichol and retinoid compounds in the stored cytosomes and indicated a possible defect in the recycling or metabolism of these compounds. These isoprenoid abnormalities are now generally regarded as secondary phenomena (Jolly, R.D., pers. comm.).

The biochemical studies on lipopigments isolated from the liver of affected sheep revealed that there was no defect in lipid metabolism or abnormal lipid peroxidation in ovine ceroid-lipofuscinosis (Palmer et al., 1986b). It was also reported by Palmer et al. (1986a) that lipopigments from affected sheep contained a high proportion of protein (approximately 70%) in which there were polypeptides of 14,800 M_r and 3,500 M_r. They are apparently specific for the disease. The latter polypeptide accounts for 71% of the protein on a molar basis and has been identified as part of the lipid binding protein of mitochondrial ATPase (Palmer, D.N.; Martinus, R.D.; Jolly, R.D., pers. comm.). The reason for its accumulation in lysosomes has not yet been elucidated. In view of these biochemical findings, it was concluded that ovine ceroid-lipofuscinosis was a lysosomal proteinosis. This hypothesis appears to be supported by the work of Ivy et al. (1984), in which they induced the formation of lysosome-associated granular aggregates which resembled the pigment "ceroid-lipofuscin", by treating young rats with the thiol protease inhibitor leupeptin.

There are a number of possible mechanisms that could account for a lysosomal proteinosis, such as deficiency of lysosomal protease or its control, secondary perturbation of lysosomal function similar to that induced by iatrogenic drugs, and the presentation to the lysosomal system of post-translationally modified protein that cannot be catabolised (Jolly et al., 1987). Another possible mechanism could be a defect in recycling from lysosomes of some specific membrane domain (Jolly et al., 1987). Evidence possibly supporting this is noted in this thesis (Fig. 4.16). Based on the biochemical studies, Wolfe et al. (1987) also considered defects in normal organellar membrane recycling as a process of lipopigment formation. In the present situation, the stored peptide is approximately half the molecular weight of the known parent protein. This suggests that ovine ceroid-lipofuscinosis may reflect a defect in the proteolysis of this protein.

Of the inherited lysosomal storage diseases, the ceroid-lipofuscinoses still remain as a group whose pathogenic pathways have not yet been elucidated. This may in part be due to a limited availability of tissues from affected human patients. However, an established flock of South Hampshire sheep carrying the ceroid-lipofuscinosis gene have made it possible to perform detailed and repetitive analytical and experimental studies on affected progeny. Results obtained from these studies have provided knowledge that should facilitate investigations into the human counter parts. The present study on the central nervous system of affected sheep has also provided critical information on the pathomorphogenesis of lipopigments which may be also relevant to that in the human syndromes. Thus, ovine ceroid-lipofuscinosis is a useful animal model for the study of the human ceroid-lipofuscinoses.

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