Ceroid-lipofuscinosis (Batten Disease)

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Abstract

The ceroid-lipofuscinoses are a group of inherited neurodegenerative diseases occurring in human beings and animals. Histologically there is generalised accumulation of a fluorescent lipopigment within the cytoplasm of many cell types and selective necrosis of some populations of neurons. Clinical signs include loss of vision, seizures and mental retardation with premature death being the eventual outcome. Systematic analyses of isolated storage material have previously resulted in the identification of subunit c of mitochondrial ATP synthase as being a major constituent in the late infantile human, ovine, bovine and three canine forms of ceroid-lipofuscinosis. Saposins A and D have been identified as being stored in the infantile human disease.

Brain biopsy has been routinely used to diagnose ceroid-lipofuscinosis in sheep. The efficacy of this technique was investigated and it was concluded that it was a safe and reliable method for diagnosis in lambs aged $2\,^{1}/_{2}$ months. In contrast, diagnosis of the disease by clinical examination could only be made comparatively late in the disease course at 9-12 months. Repeated neurological examinations enabled "clinical staging" of the progression of the disease.

Haematopoietic cell transplantation was carried out in foetal lambs with ceroid-lipofuscinosis. Stable engraftment of an average of 9% blood cells was achieved but under the conditions of the experiment there was no alteration in the disease course or severity of lesions in transplanted lambs with ceroid-lipofuscinosis as compared to affected lambs without transplanted cells.

The observation of autofluorescence from storage cytosomes in the ceroid-lipofuscinoses has lead to the assumption that they contain a fluorophore of critical significance to expla nations of pathogenenesis. Studies on the nature of the fluorescence from storage bodies and isolated storage body components allowed the conclusion that no single significant fluorophore other than protein was present.

Antibodies to subunit c of mitochondrial ATP synthase were produced. These and similar antibodies from other sources were used for immunocytochemistry. The staining pattern observed varied, depending on the fixation regime, the antibody used and the form of disease. It was concluded that different epitopes were exposed in different forms of the ceroid-lipofuscinoses. Positive immunostaining of storage material in muscle and cartilage from the ear depended on the age of the patient and could assist but not replace current methods of diagnosis. Storage cytosomes were also labelled using immunogold staining at the ultrastructural level.

The ceroid-lipofuscinoses are a genetically diverse group of diseases which appear to have complex biochemical systems underlying them. The hypothesis was developed that the defect may lie in the disassembly of the F_o complex rather than in proteolysis *per se*. The aggregation of subunit c with lipids could result in a complex structure resistant to catabolism by proteases.

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Publications

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Chapter One

General Introduction - The Ceroid-lipofuscinoses

Introduction

The ceroid lipofuscinoses are a group of inherited diseases occurring in both human beings and animals that are characterised by the generalised accumulation and storage of a fluorescent lipopigment within many cell types and selective necrosis of some populations of neurons. The clinical signs of the disease are progressive with premature death being the eventual outcome (Zeman 1976). A variety of eponymic names abound for the various forms of ceroid-lipofuscinosis reflecting the names of authors who originally described cases of these diseases (Table 1-1). These historical aspects are extensively reviewed by Zeman and Dyken (1969) Zeman (1970) and Zeman *et al* (1970).

The first detailed report of one of these diseases was by Batten (1903) who described the clinicopathological effects of the disease in two sisters beginning at 4 and 6 years of age. He later published another report containing details of an earlier onset form of the disease beginning at age $3\,^1/_2$ years in siblings from another two families (Batten 1914). Between these two reports similar disorders in children were described by Mayou (1904), Spielmeyer (1905), Vogt (1905), Jansky (1908), and Bielschowsky (1913). However, the earliest clinical description of the disease now called ceroid-lipofuscinosis was probably made by Stengel (1826; reprinted in English 1980) who described four affected siblings from a small mining community in Norway who showed progressive visual failure, speech difficulties, sensory motor regression and profound mental dullness by the age of 15 years. Although no pathological studies were performed on these children, the clinical descriptions were so succinct that a retrospective diagnosis of ceroid-lipofuscinosis was considered justified (Zeman 1976).

Classification of the Ceroid-lipofuscinoses

For over 50 years the ceroid-lipofuscinoses were combined with a variety of other diseases as the "amaurotic familial idiocies", a subgroup under the general classification of "lipidoses". The name "amaurotic familial idiocy" was originally proposed as a descriptive term for a specific disease detailed by Sachs, which later became known by the eponym Tay-Sachs disease. It became expanded to cover a wider group of diseases including at some time or another Tay-Sachs disease (GM_2 gangliosidosis), Niemann-Pick disease (sphingomyelinase deficiency), Gaucher disease (glucocerebrosidase deficiency), Hurler disease (α-L-iduronidase deficiency), Pelizaeus-Merzbacher disease (a X chromosome-linked demyelinating disease), as well as the variety of disease forms in the group now termed ceroid-lipofuscinosis (Zeman 1970). This was on the basis that the overriding unifying feature was an ubiquitous ballooning of the neuronal perikarya which later became designated as the Schaffer-Spielmeyer cell process.

In 1969 Zeman and Dyken introduced the term "neuronal ceroid-lipofuscinosis" to cover those of the original amaurotic familial idiocies where the lipopigment stored in cells had tinctorial, histochemical and fluorescent similarities to the lipopigments ceroid and lipofuscin, as distinct from the ganglioside accumulation in Tay-Sachs disease. The term amaurotic familial idiocy has now become obsolete (Zeman 1970).

Terminology for this group of diseases remains a contentious and sometimes a confusing issue. The name neuronal ceroid-lipofuscinosis is misleading since pigment storage also occurs in non-neuronal tissues, but the terminology is defended because secondary degenerative changes are limited to the nervous system. For this reason the term "ceroid-lipofuscinosis" is also used. Batten originally argued on clinical and anatomical grounds that many of the early amaurotic familial idiocies formed one entity clearly separated from Tay-Sachs disease. Although he later reversed this argument, the term Batten disease is now accepted as a generic name for this group of neurological diseases (Rider and Rider 1988).

The various disease entities within the ceroid-lipofuscinoses have remained subdivided on clinical grounds by age of onset and to a lesser degree, course of the disease. There is a general consensus that there are five major forms in human patients (Kohlschütter *et al* 1993). These are called infantile ceroid-lipofuscinosis, late infantile ceroid-lipofuscinosis, early juvenile variant ceroid-lipofuscinosis, juvenile ceroid-lipofuscinosis, and adult ceroid-lipofuscinosis. In addition a congenital form (Norman and Wood 1941, Brown *et al*

1954, Hagburg *et al* 1965, Sandbank 1968, Edathodu *et al* 1984, Humpreys *et al* 1985, Garborg *et al* 1987) and protracted juvenile form (Goebel 1993) are relatively well described. There is considerable heterogeneity within the group encompassing the ceroid-lipofuscinoses as, with the exception of the infantile form in Finnish children, the occurrence in human populations generally reflects contributions from a wide gene pool. For this reason the classification of human forms of ceroid-lipofuscinosis is complicated and a number of other variants have been described by different authors (Dyken 1988, 1989, Wisniewski *et al* 1992b, Goebel 1993).

Table 1-1: *The Classification of the Main Forms of Ceroid-lipofuscinosis by Age and Eponyms.*

Classification by Age	**Eponyms**
Infantile	Santavuori Haltia Hagberg
Late infantile	Jansky Bielschowsky
Early juvenile	Lake and Cavanagh
Juvenile	Batten Mayou Spielmeyer Vogt Sjogren
Adult	Kuf Parry Hallervorden

In infants, children and most adult cases, these diseases have an autosomal recessive mode of inheritance (Kohlschütter *et al* 1993), but in rare instances the adult form has been reported to have a dominant mode of inheritance (Boehme *et al* 1971, Arnold *et al* 1987).

Collectively the ceroid-lipofuscinoses are probably the most common world wide hereditary progressive neurodegenerative disorder in children (Dyken and Krawiecke 1983). The disease has been described in all common races and ethnic groups and shows no sex preference (Zeman and Siakotos 1973). In a recent collective study of 319 patients in the USA (Wisniewski *et al* 1992b) no patients of Afro-American or Jewish ancestry were identified. The ethnicity of most patients was caucasian, with ancestors mainly of European origin. The incidence figures vary between forms of disease from one population to another but a collective figure as high as 1 in 12,000 live births has been postulated (Rider and Rider 1988). The late infantile and juvenile forms are the most common in most populations (Wisniewski *et al* 1988, 1992b, Boustany *et al* 1988, Andermann *et al* 1988) except that in Finland the infantile disease is the most frequent form encounted with an incidence of 1 in 20,000 live births (Santavuori 1988, Santavuori *et al* 1993a).

Infantile Ceroid-lipofuscinosis

The earliest onset form of ceroid-lipofuscinosis is the infantile form (Santavuori-Haltia-Hagberg) where retardation of psychomotor development is first observed at 6 to 18 months of age. Early clinical signs include muscular hypotonia and motor clumsiness followed by severe mental retardation, myoclonic jerks, hyperexcitability and progressive visual failure over 12 to 22 months with blindness by 24 months of age. Ataxia develops and inability to walk occurs by 12 to 18 months. Epileptic seizures appear after 14 to 24 months. Patients enter a vegetative state by 3 years of age and remain in this condition until death ensues between 6 and 15 years of age (Santavuori 1988, Kohlschütter *et al* 1993, Santavuori *et al* 1993a). Neuropathological investigation shows extreme atrophy both of the cerebrum and cerebellum, and the skull bones are thickened. Histopathology of brain shows extensive accumulation of storage material and severe neuronal loss, progressively leading to almost complete loss of nerve cell bodies in the cerebral and cerebellar cortices and replacement by "glial" tissue. Large numbers of macrophages containing quantities of storage material persist in the brain, concomitant with loss of axons and myelin sheaths in the white matter (Zeman 1976). Peripheral neural and visceral cells show extensive accumulation of storage

material, without evidence of cellular destruction, except in the retina where severe atrophy is apparent (Haltia *et al* 1973).

Late Infantile Ceroid-lipofuscinosis

The late infantile form of ceroid-lipofuscinosis (Jansky-Bielschowsky) clinically presents between 2 and 4 years of age with epilepsy followed by dementia, myoclonia and ataxia. Visual deterioration occurs slowly, and death usually occurs before 15 years of age. Neuropathological findings include severe cerebellar and cerebral atrophy with severe loss of neurons, and pronounced reactive gliosis and microgliosis. Pseudolaminar cortical necrosis, with malacic clefting at the terminal stage of the disease, is reported to occur (Zeman *et al* 1970, Jolly and Palmer 1995). Storage material is also found in cells within many other body tissues (Pampiglione and Harden 1973, Harden and Pampiglione 1982, Wisniewski *et al* 1988, Zeman *et al* 1970). A variant form of late infantile ceroid-lipofuscinosis has been reported in the genetically isolated population of Finland.

Early Juvenile (Late Infantile Variant) Ceroid-lipofuscinosis

This is the most recent addition to the classification of the ceroid-lipofuscinoses (Kohlschütter *et al* 1993). The age of onset of this form is 4 to 7 years, and the age at death is 10 to 30 years. The lesions are similar to those of the juvenile form (see below) but no changes in lymphocytes are present. Mental retardation is the main presenting symptom, followed by ataxia and constant myoclonus (Lake and Cavanagh 1978, Libert *et al* 1982, Santavuouri *et al* 1982, 1991, 1993b, Wisniewski *et al* 1992b, Kohlschütter *et al* 1993).

Juvenile Ceroid-lipofuscinosis

In the juvenile form of ceroid-lipofuscinoses (Spielmeyer-Sjogren-Batten-Mayou-Vogt) the initial presenting symptom is visual impairment which develops between 4 and 9 years (Zeman 1970, Boustany *et al* 1988, Santavuori 1988, Wisniewski *et al* 1988, 1992b). Mental retardation develops insidiously and progresses slowly until dementia becomes profound late in the course of the disease. Epileptic seizures manifest between 8 and 16 years and motor dysfunction due to extrapyramidal, pyramidal and cerebellar disturbances

manifests as Parkinson-like dysfunction with inability to walk by the end of the course of disease. Some patients have a very protracted course and the age of death varies widely from 14 to 40 years. At post mortem examination the macroscopic appearance of the brain may be normal, but it is decreased in weight and the skull bones are slightly thickened. Histopathology shows neurons distended by perikaryonal granular material, but the number of neurons is usually not greatly reduced (Zeman 1976). Selective necrosis of stellate cells in layers II and III and of pyramidal cells in layer V of the isocortex is reported to occur (Braak and Goebel 1978, 1979). Astrocytes contain storage material and the white matter appears well preserved. Subtle changes including atrophy of the granular cell layer in the cerebellum may be present, but it is apparently not unusual for the cerebellum to appear normal (Zeman 1976). Storage material also accumulates in a wide variety of extraneuronal cells. Vacuolated lymphocytes are a manifestation of this storage and are of diagnostic importance, but are only seen in the classical form of juvenile ceroid-lipofuscinosis (Van Bagh and Hortling 1948).

Protracted Juvenile Ceroid-lipofuscinosis

Protracted juvenile ceroid-lipofuscinosis has been proposed as a classification for patients affected with ceroid-lipofuscinosis which become symptomatic during childhood (age of onset 6-18 years), but die as adults (age at death >32 years). The clinical symptoms show the same spectrum and order of appearance as classical juvenile ceroid-lipofuscinosis but the disease progression is well outside the normal range. This form has been reported in eight patients as summarised by Goebel (1993). However, two of these patients may have had a variant form as there was reportedly no visual impairment which is a typical presenting clinical feature in juvenile ceroid-lipofuscinosis.

Adult Ceroid-lipofuscinosis

The adult form (Kuf-Parry-Hallervorden) is considered to be the rarest form of ceroid-lipofuscinosis with an incidence between 1.3 and 10% of all cases of ceroid-lipofuscinosis (Wisniewski *et al* 1992b). This form has been reported to have two phenotypes; type A showing progressive myoclonus epilepsy, dementia and marked photosensitivity and type B showing dementia with motor disturbances and facial dyskinesia (Berkovic *et al* 1988). Visual failure is not a clinical symptom in this form of ceroid-lipofuscinosis, although Dom

et al (1979) suggest that it may be present in the late stages of the disease. In most cases the disease starts around the age of 30 years but occasionally presents during adolescence (Berkovic *et al* 1988). Onset in one patient has been reported as late as 63 years of age (Constantinidis *et al* 1992). Histopathology shows storage of autofluorescent lipopigment in neuronal cytosomes. The degree of storage is more severe than in old age (age pigment) but less severe than in juvenile ceroid-lipofuscinosis. Storage material in cells outside the nervous system has been documented in only a few reported cases of adult ceroid-lipofuscinosis. These cases report storage material in visceral tissues (Bignami *et al* 1969, Dom *et al* 1979, Constantinidis *et al* 1992) and in skeletal muscle fibres, cardiac muscle cells and in perivascular smooth muscle cells in various visceral organs (Martin 1993).

Ceroid-lipofuscinosis in Animals

Ceroid-lipofuscinosis has been described in many animal species. These include Beefmaster (Read and Bridges 1969) and Devon cattle (Harper *et al* 1988, Martinus *et al* 1991, Jolly *et al* 1992); Siamese cats (Green and Little, 1974); a Japanese cat (Nakayama *et al* 1993); South Hampshire sheep (Jolly and West 1976, Jolly *et al* 1980, 1982, 1989, Palmer *et al* 1986a, b, 1989, 1990); Swedish sheep (Järplid and Haltia 1993); Rambouillet sheep (Edwards *et al* 1994); Nubian goats (Fiske and Storts, 1988); mice *(mnd)* (Bronson *et al* 1993, Faust *et al* 1994, Pardo *et al* 1994) and many breeds of dogs (Jolly *et al* 1994). The diseases in various breeds of dogs are classified on the basis of age of onset and the course of each disease into three classes, namely prepubertal-protracted, early adult-acute and adult onset. The prepubertal-protracted form of disease occurred in a cohort of Dalmations (Malkusch 1984, Goebel and Dahme 1985, 1986, Goebel *et al* 1988). The early adult-acute course disease is described in English setters (Koppang 1970, 1973/1974, 1987, 1988, 1992, Armstrong *et al* 1982); Border collies (Taylor and Farrow 1988a, 1992, Studdert and Mitten 1991); Salukis (Appleby *et al* 1982); Blue heelers (Cho *et al* 1986, Wood *et al* 1987, Sisk *et al* 1990); Chihuahuas (Rac and Giesecke 1975, Jolly and Hartley 1977); Golden retrievers (Patterson in Jolly *et al* 1994); Pekingese (Kelly in Jolly *et al* 1994); Spitz (Pickett *et al* 1992); a "golden spaniel" (Fankhauser 1965) and a Yugoslavian shepherd (Bichsel and Vandevelde 1982).

Adult forms of ceroid-lipofuscinosis have been described in Tibetan terriers (Cummings and de Lahunta 1977, Riis and Loew 1988, Cummings *et al* 1991, Alroy *et al* 1992, Riis *et al* 1992); Corgis (Kelly in Jolly *et al* 1994); a wire haired Dachshund (Cummings and

deLahunta 1977); long-haired Dachshunds (Vandevelde and Fatzer 1980); Cocker spaniels (Hänichen and Püschner 1971, Nimmo Wilkie and Hudson 1982, Jolly *et al* 1994); a Springer spaniel (Fankhauser 1965); a Terrier cross dog (Hoover *et al* 1984) and in miniature Schnauzers (Sutton in Jolly *et al* 1994).

Genetic information showed the disease to be inherited as a Mendelian recessive in English setter (Koppang 1973/1974) and Border collie dogs (Studdert and Mitten 1991), and in Rambouillet (Edwards *et al* 1994) and South Hampshire sheep (Jolly *et al* 1980, 1982). Anecdotal evidence suggests similar inheritance for the disease in Swedish sheep, Devon cattle, Nubian goats and Tibetan terrier and Saluki dogs. In most other animal species too few cases have been recorded to confirm the mode of inheritance but there is no reason from the reports available to suggest other than autosomal recessive inheritance. Ceroid-lipofuscinosis in South Hampshire sheep has been extensively studied and is reviewed below.

Ceroid-lipofuscinosis in South Hampshire Sheep

Ceroid-lipofuscinosis was first diagnosed in an inbred flock of South Hampshire sheep on clinical and histopathological grounds (Jolly and West 1976). The South Hampshire breed was developed by crossing Southdown and Hampshiredown breeds followed by inbreeding to "fix type". An experimental flock has been established and maintained as an animal model for the human diseases (Jolly *et al* 1980, 1982, 1988, 1989, 1990). Affected lambs appeared normal from birth to 7 months when neurological signs were first noted (Mayhew *et al* 1985). These signs included a delayed response to the presence of an observer, and a depressed eye preservation (menace) response. When travelling, affected sheep held their heads high and tilted them to move from one visual field to another. These sheep tended to graze alone, lag behind the flock when moved, and became difficult to control with a sheep dog. Around 8 months of age, sheep started to move with their heads low to the ground, particularly when passing through doorways or along corridors. By 9 months of age visual deficit was evident with loss of the "menace" response, baulking at gateways, drains or shadows and depressed pupillary reflexes and "blink to bright light" response. After 10 months to 1 year, lambs with ceroid-lipofuscinosis were smaller than normal. Behavioural changes became more evident with intermittent, vigorous and unpredictable struggling during normal restraint in a sitting position. There were spontaneous episodes of head nodding, jaw champing and twitching of ears, eyelids, lips and muzzle. These episodes

increased in severity when the animals were disturbed or excited. After 18 months of age, sheep were depressed and appeared almost oblivious to their environment. "Menace" and "blink to bright light" responses were absent, pupils were widely dilated and pupillary reflexes were very sluggish although present. No abnormalities were detected by examination of the fundus. Animals wandered aimlessly, often in circles. They spent considerable time eating and drinking and the wool of the lower jaw was constantly wet from periods of sham drinking (Jolly *et al* 1980). Episodes of tremor of the face, head, neck and limbs occurred intermittently, but this was controlled by phenobarbitone and diazepam suggesting that they were partial seizures that did not generalise as sheep have a high seizure threshold (Mayhew *et al* 1985). Sheep have lived up to 29 months of age but were usually euthanased on humane grounds prior to this.

Gross Pathology and Histopathology of Ovine Ceroid-lipofuscinosis

In the affected sheep with ceroid-lipofuscinosis the only gross pathological change noted at necropsy was cerebral atrophy (Jolly *et al* 1980, 1982, 1989, Mayhew *et al* 1985). There was essentially no difference in brain weights between lambs affected with ceroid-lipofuscinosis and normal lambs at birth but brains from affected lambs increased in weight more slowly than normal brains and began to exhibit atrophy from 4 to 6 months of age. Brains from affected sheep were approximately half the weight of normal brains when the sheep were 25 - 29 months of age. Atrophy was mainly associated with the forebrain with much less effect on the brainstem and cerebellum. The brain texture was firmer than normal and the lateral ventricles were slightly enlarged and there was thinning of the *septum pellucidum* and *corpus callosum.*

Histopathology of atrophic brains showed severe and obvious loss of neurons in the cerebral cortex (Jolly *et al* 1980, 1982, Mayhew *et al* 1985). This loss occurred in a laminar pattern accompanied by a fibrillary astrocytosis that was evident from 10 weeks of age in affected lambs (Jolly *et al* 1989). These changes became severe until in the terminal phase of the disease, i.e. from 25 months, relatively few neurons remained. The observed astrocytosis had three components; hyperplasia, hypertrophy and condensation of tissue associated with neuronal loss and the presence of increased numbers of lipopigment-laden macrophages. Changes in the white matter consisted of Wallerian-type degeneration and a fibrillary astrocytosis that was less severe than in the grey matter. Autofluorescent storage bodies

ranging from < 0.5 μm up to 15 μm in diameter were seen in neurons and many other cell types within the body.

General Histochemical Staining and Fluorescence of Storage Bodies in the Ceroid-lipofuscinoses

There is a general similarity in the histochemical staining and fluorescence properties of storage bodies in all forms of the disease that essentially defines them as belonging within the group termed ceroid-lipofuscinosis (Jolly and Palmer 1995). Characteristically storage bodies show yellow autofluorescence under blue light (440-590 nm). This autofluorescence was comparatively mild in the congenital form in Swedish sheep (Jolly and Palmer 1995).

Ceroid-lipofuscinosis storage bodies do not dissolve in the aqueous or organic solvents used in histological slide processing and thus remain intact in paraffin sections. With haematoxylin and eosin (H&E) staining, the bodies appear refractile and slightly eosinophilic. The storage material stains strongly with fat stains such as Sudan black and Sudan IV but less intensely with Sudan III or oil-red-O. The staining is generally positive with the periodic acid Schiff (PAS) method although this can be variable, particularly outside the nervous system. In most forms of ceroid-lipofuscinosis storage cytosomes in nervous tissue stain strongly with Luxol fast blue (LFB), but the degree of staining varies widely in other tissues. Staining with LFB is slight or absent in the infantile human (Lake 1984), adult miniature Schnauzer dog and congenital form in Swedish lambs (Jolly and Palmer 1995). The material in storage bodies is unstained by Schmorl's stain, which stains both melanins and lipofuscins in aged tissues (Stevens 1982), or the Masson-Fontana method which is commonly used to stain melanins but may stain some lipofuscins (Stevens 1982).

Ultrastructure of Storage Cytosomes

In the 1960's much attention was given to the electron microscopic observations of unique cytosomes in lysosomes within the nervous system (Gonatas *et al* 1963, Zeman and Donahue 1963, Suzuki *et al* 1968), and within non-neuronal tissues. These cytosomes consisted of membrane bound profiles commonly described as membrane bound granular osmophilic deposits (GROD), or "Finnish snowballs", pure granular profiles, fingerprint bodies or curvilinear bodies, rectilinear bodies and lamellar profiles (Lake 1984). It was

concluded that ultrastructural analysis of storage bodies promised little insight into the biochemical nature of the ceroid-lipofuscinosis disease group (Goebel *et al* 1979). In the 1970's efforts were made to classify the various ceroid-lipofuscinosis disorders on the basis of these ultrastructural profiles but these have proved to be controversial. Some studies have claimed that all the different profiles may be seen in all clinical forms of the disease (Zeman *et al* 1970, Goebel *et al* 1979, Rapola 1993). In contrast a recent study by Wisniewski *et al* (1992b) claimed that the ultrastructure of bodies present in skin or conjunctiva punch biopsies and/or in buffy coat leucocytes are of nosological significance. Granular osmiophilic profiles were found in 36 infantile cases, curvilinear profiles in 116 late infantile cases and fingerprint profiles in 163 juvenile cases of ceroid-lipofuscinosis. Three adult cases showed fingerprint and curvilinear profiles, while one other adult case showed no characteristic changes in extraneuronal tissues.

Biochemical Basis of the Ceroid-lipofuscinoses

The underlying metabolic defect in all forms of ceroid-lipofuscinosis remains unknown. From the histological staining and fluorescent properties of storage material a defect in lipid metabolism was assumed and historically the ceroid-lipofuscinoses have been strongly connected with the lipidoses. Model studies by Chio and Tappel (1969a, b) reported that malonaldehyde, which is a product of lipid peroxidation, reacted with proteins or phospholipids to generate Schiff bases with the same fluorescent properties as lipofuscin. As a direct result of these studies it was postulated that the primary defect involved in the formation of storage material was a defect in the metabolism of polyunsaturated fats (Zeman and Dyken 1969, Zeman 1974, Zeman 1976). Support for this hypothesis came from Armstrong *et al* (1973) who proposed a deficiency in leucocyte peroxidase in a patient with late infantile ceroid-lipofuscinosis and two patients with the juvenile form of the disease. Numerous other studies appeared over the next few years affirming this leucocyte peroxidase abnormality, but other studies questioned these results and the methodology of measurement which proved to be inappropriate and inaccurate (Parry *et al* 1976).

The first analytical studies on the material stored in the ceroid-lipofuscinoses were carried out by Siakotos and colleagues (Siakotos *et al* 1972, Siakotos and Koppang 1973, Siakotos *et al* 1978, Siakotos and Munkres 1981). They isolated two fluorescent substances. The first was called an "acidic fluorophore" and was from the lipid components in storage material and believed to be a highly fluorescent Schiff base polymer formed as a consequence of lipid

peroxidation. The second was a fluorescent polymer found in the neutral lipids that was subsequently designated as "polymalonaldehyde" on the basis of its chromatographic and fluorescent properties. A number of hypotheses concerning pathogenesis of pigment formation developed from the lipid peroxidation theory (Table 1-2 *Part A*) but these have not stood the tests of time or experimentation. Other hypotheses not involving lipid peroxidation have also been developed (Table 1-2 *Part B*).

Table 1-2: *Hypotheses Concerning the Pathogenesis of the Ceroid-lipofuscinoses.*

Part A: *Hypotheses Concerning Lipid Peroxidation.*

Proposed defect	**Reference**
Abnormal lipid peroxidation	Zeman 1974 Siakotos *et al* 1972 Siakotos and Koppang 1973 Siakotos and Munkres 1981 Siakotos *et al* 1988,1993
Peroxidase deficiency	Armstrong *et al* 1973
Disturbances of fatty acid metabolism	Svennerholm *et al* 1975 Pullarkat *et al* 1982
Disturbances in iron metabolism	Gutteridge *et al* 1982a, b

Table 1-2: *Hypotheses Concerning the Pathogenesis of the Ceroid-lipofuscinoses.*

Part B: *Hypotheses not involving Lipid Peroxidation.*

Proposed defect	**Reference**
Defects in retinoic acid and dolichol metabolism	Wolfe *et al* 1977 Ng Ying Kin *et al* 1983
Defective metabolism of dolichol-linked oligosaccharides	Hall and Patrick 1985
Lack of cathepsin D activity	Pullarkat *et al* 1988
Defective thiol endoprotease	Wolfe *et al* 1987
Inhibition of cathepsin B activity by accumulating abnormal peroxides	Dawson and Glaser 1988
Defective very low density lipoprotein synthesis	Gillis *et al* 1987
Defective processing of amyloid precursor protein	Wisniewski and Maslinska 1989
Abnormal protein s-methylated methionine	Katz and Gerhardt 1990
Abnormal protein trimethyllysine	Katz and Rodrigues 1991 Katz and Gerhardt 1992
Reduced lysosomal phospholipase A1 activity	Dawson *et al* 1993
Deficient mobilisation of calcium	Bennett *et al* 1993

A defect in dolichol metabolism was suggested as a possible cause of ceroid-lipofuscinosis as the dolichol content of brain tissue was shown to be increased in the infantile, late infantile and juvenile forms of disease (Ng Ying Kin *et al* 1983, Wolfe *et al* 1981, 1983, Hall and Patrick 1988, Hall *et al* 1989). Studies on dolichol metabolism in fibroblasts in cell culture failed to show any such abnormalities (Paton and Poulos 1984). Increased concentrations of dolichol in cerebral cortex have also been found in patients with Alzheimer disease, in aged individuals (Wolfe *et al* 1982a, b, Pullarkat and Reha 1982, Ng Ying Kin *et al* 1983) and in tissues from other inborn errors of metabolism involving lysosomal proliferation (Hall *et al* 1989). These findings suggest that alterations in dolichol concentrations may be a secondary phenomenon.

Higher than normal concentrations of phosphorylated dolichol-containing compounds, largely in the form of dolichyl pyrophosphoryl oligosaccharides, were found in brains of patients with infantile, late infantile and juvenile ceroid-lipofuscinosis (Hall and Patrick 1985, 1988, Pullarkat *et al* 1988, Hall *et al* 1990, Daniel *et al* 1992). Consequently, a defect in the metabolism of dolicholpyrophosphate-linked oligosaccharides involved in the glycosylation of proteins was suggested as a possible biochemical basis for the disease (Pullarkat 1990, Pullarkat and Zawitosky 1993). However, Andersson *et al* (1987) found that phosphorylated dolichol concentrations also increased with age. Wolfe *et al* (1988a, b) demonstrated increased dolichyl phosphate concentrations in brains from patients with Alzheimer disease, GM_1-gangliosidosis and GM_2-gangliosidosis indicating increased brain phosphorylated dolichol concentrations are not unique to the ceroid-lipofuscinoses. Hall *et al* (1992) showed that the synthesis and turnover of dolichyl phosphate appeared to be normal in fibroblasts cultured from juvenile Batten disease patients. Quantitative analysis showed that phosphorylated dolichol-containing compounds isolated from storage cytosomes in each childhood form of Batten disease accounted for 7% or less of the cytosome dry weight. In sheep these compounds comprised 1-2% of the dry weight of storage cytosomes isolated from brain and pancreas and 0.5% and 0.1% respectively of storage cytosomes isolated from liver and kidney (Hall *et al* 1989).

Immunocytochemical studies have implied that ceroid-lipofuscinosis may involve abnormal processing of β-amyloid precursor protein (Wisniewski and Maslinska 1989, Wisniewski 1990a, b, Kitaguchi *et al* 1990, Wisniewski and Kida 1990, Wisniewski *et al* 1992a, 1993). This protein is a membrane glycoprotein involved in the regulation of cell growth and neuronal development. The suggestion that this protein may be abnormally processed in Batten disease is of potential importance to its pathogenesis as Yankner *et al* (1989)

proposed that some fragments of β-precursor protein may be neurotoxic for animal neurons in tissue culture. However, strong immunostaining of cells has also been observed in some cases of mucopolysaccharidosis. No quantitative biochemical studies have been reported and the significance of the immunocytochemical results to the ceroid-lipofuscinoses remains to be determined.

Apart from the earlier work of Siakotos and colleagues (see above) the only complete analytical studies on ceroid-lipofuscinosis material were carried out by Palmer and colleagues. Palmer *et al* (1985) investigated the phospholipid fatty acid compositions in brain grey matter from ceroid-lipofuscinosis affected sheep and found that they were similar to those of normal sheep. Sheep are ruminants and must conserve their restricted essential fatty acids for structural functions so any defect in fatty acid metabolism should be reflected in phospholipid fatty acid compositions. This was not the case, so it was concluded that the pathogenesis of the disease did not involve any abnormality in fatty acid metabolism. These conclusions are supported by the findings in Zellwegers syndrome (failure of peroxisome biogenesis) where defective oxidation of very long chain fatty acids is present. The clinical and pathological features of this syndrome were dissimilar to those observed in Batten disease (Lazarow and Moser 1989).

Further studies by Palmer *et al* (1986a, b, 1988) were based on the assumption that the nature of the lipopigment stored within cells must in some way reflect the underlying biochemical lesion. To avoid the complication of secondary changes (e.g. brain atrophy and associated demyelination) or changes in lipids due to lipopigment aging in post-mitotic neurons, visceral lipopigment extracted from liver, pancreas and kidney was used. The major component of the storage bodies was initially shown to be protein by elemental analysis and amino acid analysis of the residue remaining after extraction of the lipopigment lipids. Gravimetric analysis showed this proteinaceous component comprised between 64% and 76% of the total lipopigment mass. Silver staining of lipopigment proteins separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed two major protein bands with apparent molecular weights of Mr 3,500 and Mr 14,800. The dominant band was the Mr 3,500 protein. These were not normal lysosomal proteins and they were relatively insensitive to Coomassie blue and Amido black stains.

The other major component in the disease related storage material was lipid (Palmer *et al* 1986a). These consisted of 50% phospholipids and 50% neutral lipids. The phospholipids were those found in normal mammalian membranes including phosphatidylcholine,

phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol and an additional known lysosomal marker bis (monoacylglycero) phosphate. Neutral lipids in the lipopigment were ubiquinone, dolichyl esters, dolichol, free fatty acids and cholesterol (Palmer *et al* 1986a). These lipids and 1-2% metals were indicative of the lipopigment cytosomes functioning as lysosomes at some stage in their biogenesis (Palmer *et al* 1988). No significant differences were found between the lipids of normal and ovine ceroid-lipofuscinosis affected livers or in their fatty acid profiles, so there was no evidence for an abnormality in phospholipid or polyunsaturated fatty acid metabolism in this disease.

Isolated storage bodies retained the characteristic fluorescent properties observed *in situ*; however the fluorescence was lost on separation into various components. Extracted lipids from storage bodies contained a number of weak fluorophors, but none of these was a major lipid component or could be equated with a putative Schiff base polymer consequent to lipid peroxidation (Palmer *et al* 1986a).

Protein sequencing of purified total lipopigment proteins revealed a dominant sequence identical to the NH_2 terminus of the highly hydrophobic dicyclohexylcarbodiimide (DCCD) binding proteolipid of proton translocating mitochondrial ATP synthase (Palmer *et al* 1989). This 75 residue protein is highly conserved between species and has a Mr of 7608 in mammals (Sebald *et al* 1979, Gay and Walker 1985). It is also known as subunit c, subunit 9 or the lipid-binding subunit of mitochondrial ATP synthase. The term proteolipid derives from the lipid-like solubility of this and other proteins in chloroform/methanol mixtures (Folch and Lees 1951), but there is no implication of covalently bound lipid. Subunit c is highly hydrophobic and consequently has a high detergent to protein ratio in micelles for polyacrylamide gel electrophoresis (PAGE), hence the apparent molecular weight of 3.5 kDa (Palmer *et al* 1988, 1992). Sequencing of a higher Mr storage body component, at an apparent molecular weight of 14.8 kDa after PAGE, showed this band was an aggregate of subunit c (Fearnley *et al* 1990).

The major stored component in ovine ceroid-lipofuscinosis has been shown to be complete subunit c and from sequencing it has been shown to be identical to subunit c as it is assembled into the mitochondrial ATP synthase complex. Mitochondrial import sequences present at the N-terminal end of precursors of subunit c have been removed (Palmer *et al* 1988, 1990, Fearnley *et al* 1990). The mature subunit c is not cleaved in any way and there was no evidence of N or C terminal cleavage or "raggedness".

The N-terminal sequence of mitochondrial ATP synthase subunit c has also been found to be present in storage body preparations from juvenile and late infantile forms of the human disease (Palmer *et al* 1990, 1992), in a Devon heifer (Martinus *et al* 1991), and in English setter, Border collie and Tibetan terrier dogs (Jolly *et al* 1992, Palmer and Jolly pers. comm.). Immunocytochemical studies suggested that subunit c is also stored in Kuf disease (adult ceroid-lipofuscinosis) (Hall *et al* 1991a, Westlake *et al* 1995, see chapter seven). In contrast, subunit c was not detected in storage material in the infantile form of Batten disease (Palmer *et al* 1990, 1992, Ezaki *et al* 1993) but saposins A and D were shown to accumulate (Tyynelä *et al* 1993).

Subunit c is an integral inner membrane component of the membrane bound F_0F_1-ATP synthase complex of mitochondria, chloroplasts and bacteria. This ATP synthase complex catalyses ATP production from ADP and inorganic phosphate. The energy for this comes from the coupled release of a proton across the transmembrane potential gradient (Issartel *et al* 1992). The enzymes from various sources differ in the complexity of their subunits. The simplest is that from *Escherichia coli* (Fillingame 1981) which has at least eight different subunits. Mitochondrial ATP synthases are even more complicated and contain at least 16 different polypeptides (Collinson *et al* 1994). Each complex contains several copies of some of these polypeptides including multiple copies of subunit c (Foster and Fillingame 1982).

In mammals subunit c is coded for on nuclear genes. Initially two genes called P1 and P2 were described (Gay and Walker 1985, Dyer *et al* 1989) and recently a third gene called P3 has been demonstrated (Yan *et al* 1995). These genes encode for the same mature protein but have different N-terminal presequences which direct the importation of mature protein into mitochondria. The presequences are removed during or shortly after the import of subunit c into the mitochondria (Schmidt *et al* 1984, Pfaller *et al* 1988, Hartl *et al* 1989, Hendrick *et al* 1989).

The sequences of the P1 and P2 gene products including lead sequences from affected and normal sheep have been determined from cDNA libraries using the polymerase chain reaction (PCR) technique. These gene products were identical in affected and normal sheep, thus the disease is not caused by mutations in the mitochondrial import sequences (Medd *et al* 1991, Palmer *et al* 1990, Medd *et al* 1993). Northern blot hybridisation showed no gross differences in the concentrations of the mRNAs for P1 and P2 between affected and normal sheep, hence subunit c is not synthesised in greater amounts in diseased animals. This

implied that the accumulation of subunit c in lysosomes in ceroid-lipofuscinosis was not due to overloading of the mitochondrial import pathway (Medd *et al* 1993).

Medd *et al* (1993) isolated a mutated cDNA related to P2 from the gene library derived from a sheep with ceroid-lipofuscinosis. This raised the possibility that the pseudogenes were linked to the genetic cause of the disease. However, restriction analysis of the genomic DNA from an additional 14 sheep showed that the different pseudogenes were not linked to the gene causing ceroid-lipofuscinosis.

Additional evidence for the exclusion of a P1 or P2 gene-related cause in the ceroid-lipofuscinoses comes from gene linkage studies. The gene for the juvenile form of human ceroid-lipofuscinosis maps to the long arm of chromosome 16 (Gardiner 1992, see below), whereas human P1 and P2 genes are on human chromosomes 17 and 12 respectively (Dyer and Walker 1993).

It appears that mature subunit c is processed normally into the inner mitochondrial membrane. It does not accumulate there as LDS-PAGE preparations of inner mitochondrial membranes from both affected and normal sheep showed barely detectable but similar amounts of subunit c (Fearnley *et al* 1990). Oxidative phosphorylation was measured using isolated kidney mitochondria and was found to be as tightly coupled from affected sheep as in those from normal sheep (Palmer *et al* 1991). There is no evidence for disruption of mitochondrial function at least in regard to oxidative phosphorylation. Taken together, all the experimental evidence thus far indicates that ovine ceroid-lipofuscinosis is the result of a lesion in a specific catabolic pathway for subunit c. Jolly *et al* (1995) extended this hypothesis to include the possibility that association of subunit c with lipids may form a complex which is insusceptible to the normal cell turnover mechanisms of either of these constituents separately. Very little is known about the catabolism of mitochondrial proteins, only that lysosomes digest whole mitochondria sequestered by autophagy. Lysosomal proteases, called cathepsins, have a wide range of specificities (Luzikov 1985, Pfeifer 1987). However, the specificity of storage which occurs and the observation that the degradation rates of inner mitochondrial membrane proteins vary greatly (Hare 1990) suggest that there are specific pathways by which these proteins are catabolised that have yet to be described.

A minor protein sequence was found in ovine pancreatic storage bodies, additional to subunit c (Palmer *et al* 1989). This was homologous with amino acids 7 - 17 of a proteolipid

isolated from bovine chromaffin granule H^+-ATPase known as vacuolar ATPase subunit c (Mandel *et al* 1988) and to the N-terminal sequence of a 17 kDa protein isolated from mouse gap junction preparations (Walker *et al* 1986). The significance of this minor sequence is not known (Palmer *et al* 1990).

Biochemical studies on *mnd* mice, originally described as a model for motor neuron degeneration, have shown that subunit c of mitochondrial F_1F_0-ATP synthase and another related proteolipid, subunit c of vacuolar H^+-ATPase accumulate within storage bodies from a variety of tissues (Faust *et al* 1994). Subunit c of mitochondrial F_1F_0-ATP synthase was reported to be dramatically increased in brain, kidney, liver, heart and pancreas, while subunit c of vacuolar H^+-ATPase was shown to occur in lesser amounts in brain, kidney and liver, and was not detected in heart or pancreas. Aged mice and mice from other mutant lines (juvenile bare and mucopolysaccharidosis type VII) did not accumulate either of these proteolipids. Dolichol pyrophosphate-linked oligosaccharides were also shown to be increased 17-fold in *mnd* mouse brain.

Subunit c in storage bodies has been reported to contain high levels of S-methyl methionine (Katz and Gerhardt 1990) and trimethyllysine (Katz and Rodrigues 1991). S-methyl methionine was identified after isolation by thin layer chromatography (TLC) analysis of acid digests of brain storage material and mass spectral analyses. Trimethyllysine was identified after TLC analysis of acid digests of storage material from both retina and cerebral cortex, based on chromatographic comparison with an authentic trimethyllysine standard. It was also identified in the protein hydrolysates from ceroid-lipofuscinosis affected tissues by standard amino acid analysis. Based on staining with ninhydrin of TLC and on quantitative amino acid analysis, it was estimated that trimethyllysine represented approximately 1 mol% of the amino acids in the total hydrolysate of partially purified storage bodies from retina, and about 0.7 mol% of the amino acids from cerebral cortex storage body preparations. These results were interpreted to indicate that in both tissues approximately 20% of the total storage body protein lysine residues were methylated. The hypothesis that this is disease-related has not been substantiated due to the lack of data from appropriate normal control mitochondrial preparations. The presence of trimethyllysine has been confirmed in ovine storage bodies (Palmer *et al* 1993) but amino acid analyses from normal sheep liver mitochondria showed that these also contained a similar amount of trimethyllysine.

Genetic Linkage Studies in the Ceroid-lipofuscinoses

The gene locus in infantile ceroid-lipofuscinosis has been assigned to the vicinity of the *L-myc* oncogene at 1 p 32 (a region on the short arm of chromosome 1) using random linkage studies (Järvelä *et al* 1992, Vesa *et al* 1995). Prenatal and carrier diagnosis by linkage analysis were reported to be highly reliable in infantile ceroid-lipofuscinosis families (Vesa *et al* 1995). The gene for juvenile onset Batten disease has been localised by genetic linkage analysis to the interval between loci D16S297 and D16S57 on chromosome 16 p12.1-11.2 (Eiberg *et al* 1989, Gardiner *et al* 1992, Callen *et al* 1991, Mitchison *et al* 1994). That for the Finnish late infantile variant form has been assigned to chromosome 13q31-32 (Savukoski *et al* 1995). The gene locus for late infantile ceroid-lipofuscinosis remains unknown, although linkage analysis has excluded it from the infantile, juvenile, and Finnish late infantile variant loci (Savukoski *et al* 1995, Sharp *et al* 1995).

Chapter Two

Haematopoietic Cell Transplantation in Genetic Diseases - Review

Introduction

The concept of transplantation of body parts or tissues has existed within mythology for centuries. There are chimeric legendary characters in many cultures, and there is logic in the concept of replacing a diseased or defective tissue or organ completely with a healthy one. During the past three decades the collective body of knowledge on transplantation and immunity has burgeoned, to the extent that transplantation of body parts and tissues has become a practical reality.

The rationale for the use of cell transplantation in genetic diseases developed from work carried out in the 1960's-70's on metabolic collaboration in fibroblast cultures from patients with Hurler, Hunter and Sanfilippo forms of mucopolysaccharidosis (MPS types I, II and IIIA). This work showed that correction of aberrant patterns of mucopolysaccharide metabolism occurred when defective fibroblasts were cultured with *(a)* normal fibroblasts, *(b)* fibroblasts from patients with differing syndromes, *(c)* concentrated media in which a different fibroblast type had been grown, *(d)* normal human urine or *(e)* homogenates of connective tissue such as newborn mouse skin and bovine sclera (Fratantoni *et al* 1968, Neufeld and Fratantoni 1970, Neufeld and Cantz 1971, von Figura *et al* 1975).

Enzyme Replacement Therapy

Research involving the culture of fibroblasts as noted above, stimulated a direct approach to therapy of various enzyme deficiencies by attempting to replace the deficient enzyme. In these studies, enzyme replacement therapy was attempted using repeated infusions of plasma, whole blood, leukocytes, or enzyme purified from various sources. Such attempts at treatment were mainly on patients with different forms of mucopolysaccharidosis (Di Ferrante *et al* 1971, Dean *et al* 1973, Knudson *et al* 1971, Moser *et al* 1974, Nishioka *et al*

1979). Initially the results of this enzyme replacement appeared promising in heritable metabolic disorders where there was no central nervous system involvement such as in Fabry disease (Brady *et al* 1973) and Gaucher disease (Brady *et al* 1975). However, the outcome of longer term studies did not fulfil earlier hopes and the results were mainly equivocal (Desnick *et al* 1976, Tager *et al* 1980).

Enzyme replacement by intravenous infusion of glucocerebrosidase in Gaucher disease was investigated, initially with discouraging results (Beutler *et al* 1977, Beutler 1981, Brady *et al* 1974). However, a biotechnology firm was able to purify large amounts of glucocerebrosidase and sequentially deglycosylate the oligosaccharide chains of the native enzyme to permit efficient delivery of the enzyme to the lysosomes of macrophages. Treatment with this enzyme by intravenous infusion is reported to be safe and effective in non-neuronopathic Gaucher disease (Barton *et al* 1990, 1991, Brady *et al* 1990). This product is now commercially available.

Treatment by intravenous infusion of purified enzyme appears unsuitable for inherited metabolic diseases with central nervous system manifestations (Erbe 1974, Brady *et al* 1975). Neurons in the central nervous system are relatively inaccessible due to the blood-brain barrier preventing entry of enzyme and they have a low endocytic rate (Ohata *et al* 1990). Alteration of the blood-brain barrier was undertaken in an attempt to increase enzyme uptake by nervous tissue. This was performed by exposure to hyperbaric oxygen immediately after enzyme injection in cats (Rattazzi *et al* 1980), or by the administration of arabinose or mannitol in rats and monkeys (Barranger *et al* 1980). Intrathecal injection of enzyme in humans has been attempted in metachromatic leukodystrophy (Greene *et al* 1969) and in Tay-Sachs disease (Godel *et al* 1978, von Specht *et al* 1979). No beneficial effect was reported in any of these studies, possibly due to low neuronal uptake by endocytosis.

It has been reported that effective uptake and delivery of an exogenous lysosomal enzyme to lysosomes is possible in cultured neurons after conjugation of the enzyme with tetanus toxin fragment C (Dobrenis *et al* 1992). Cerebral cortex cell cultures from GM_2 gangliosidosis cats were exposed to tetanus toxin fragment C conjugated hexosaminidase A and ganglioside storage was virtually eliminated. In contrast, only a minimal effect on ganglioside storage was shown after exposure of identical cultured cells to hexosaminidase A enzyme alone. Further studies in this area are required before this vector system becomes clinically applicable.

A logical advancement in the area of enzyme replacement therapy appeared to be the use of carrier systems to deliver exogenous enzymes to where they are needed and to prolong the latency of these enzymes. Phospholipids can form closed concentric bilayer membranes (liposomes or vesicles) and almost any substances can be incorporated into these as long as their formation is unimpeded. Liposomes have been tried as vehicles for enzyme replacement therapy (Hudson *et al* 1980). However liposomes, instead of preventing immune response to entrapped enzymes, can act as immunologic adjuvants (Allison and Gregoriadis 1974), enhancing both humoral and cell-mediated immunity (Manesis *et al* 1979). The application of liposomes as carriers in enzyme replacement therapy did not produce any significant improvement in clinical symptoms in the patients treated and enzyme delivered in this manner proved to be highly antigenic (Gregoriadis *et al* 1980, Hudson *et al* 1980).

Tissue Transplantation

Attempts were made to overcome the difficulty of a short half life of exogenous enzymes by transplanting skin (Dean *et al* 1975) or cultured fibroblasts (Dean *et al* 1976, 1979). The transplantation of HLA-compatible cultured fibroblasts was attempted in patients with Hunter disease (MPS type II) (Dean *et al* 1976) and Sanfilippo A syndrome (MPS type IIIA) (Dean *et al* 1981). In both cases the fibroblasts were reported to survive and release the required lysosomal enzymes increasing the catabolism of accumulated sulphates or glycosaminoglycans, and as a result it was reported that the evolution of the clinical syndromes had been slowed, at least in the short term. Other workers (Purkiss *et al* 1983) involved in long term studies concluded that fibroblast transplantation does not change the clinical syndromes observed. They raised the possibility that a short term effect of the immunosuppressive drugs was to increase the excretion of low molecular mass glycosaminoglycans at the expense of the higher molecular mass fraction.

Human amniotic epithelial cells have been reported to be a good source of lysosomal enzymes that do not stimulate an immunogenic response (Akle *et al* 1981, Adinolfi *et al* 1982, Scaggiante *et al* 1991). Therapeutic trials of amniotic tissue transplantation have been carried out in patients with I-cell disease, Tay-Sachs disease, Gaucher disease, metachromatic leukodystrophy, Farber disease, GM_1 gangliosidosis and various forms of mucopolysaccharidosis (Muenzer *et al* 1985, Tylki-Szymanska *et al* 1985, Yeager *et al* 1985, Sakuragawa *et al* 1992). Despite advantages such as no graft-versus-host reaction and a readily available source of tissues, the procedure proved to be of questionable value for

clinical use. Improvement in disease status of some patients after treatment was reported but the epithelial cells had a limited survival time necessitating repeated implantation. As amniotic tissue contains a small number of fibroblasts and mononuclear cells which may stimulate a local immunogenic reaction with repeated implantation, epithelial cells from primary cultures were considered a more efficacious source of enzyme (Sakuragawa *et al* 1992).

Organ Transplantation

The modern era of pharmacologic immunosuppression was initiated by Schwartz and Dameshek's (1959) application of immunosuppressant drugs. Immunosuppression coupled with the recognition of histocompatability antigens enabled whole organ transplantation to become available as a therapeutic option. Organ transplantation is a potential cure for inherited diseases where it is sufficient to replace diseased organs or provide a source of exogenous enzyme without curing the metabolic defect. There are some serious limitations to organ transplantation. Firstly obtaining fresh, biologically functional organs is a major problem and while dramatic progress has been made in the area of immunosuppression, the survival of the organs is limited in many cases (Terasaki 1991). The surgery involved is complicated and expensive and can only be performed at relatively few locations.

Uraemia is the primary indication for renal transplantation in Fabry disease but the results gained in transplant patients have been equivocal (Van den Bergh *et al* 1976). Splenic and renal transplantation have been attempted in patients with Gaucher disease (Groth *et al* 1971, 1979). The results of these transplants are difficult to interpret due to rejection problems and difficulty in assessing the activity of the enzyme supplied. Liver transplantation has been reported in Gaucher disease, not as a treatment, but as a tool in the management of the hepatic complications (DuCerf *et al* 1992). Whole organ transplantation is successful in the treatment of some genetic diseases such as cystic fibrosis (Whitehead *et al* 1991). Other metabolic diseases however required a population of permanent, self replicating normal enzyme producing cells capable of whole body distribution, such as is the case with bone marrow transplantation.

Haematopoietic Cell Transplantation

Bone marrow transplantation by infusion became a practical manoeuvre around 1956-60 with the development of reliable methods for collecting, defatting, filtering and storing bone marrow. However, by 1966 it was found that only grafts from closely matched individuals, such as identical twins, were readily successful. Total body irradiation could ensure engraftment of bone marrow from almost any donor but graft-versus-host disease killed the recipients in most cases. The development of mixed lymphocyte culture to check the genomic compatibility of recipient and donor made bone marrow transplantation a viable therapeutic option for some diseases. In principle, marrow transplantation can be expected to ameliorate any enzyme-deficient state provided that enzyme-competent cells, or enzyme released from them, circulate to sites of disease and are present at physiologically relevant concentrations.

The first cure of a genetic disease using bone marrow transplantation was reported in 1968 (Gatti *et al* 1968). The patient was a boy afflicted with sex-linked severe combined immunodeficiency disease who was successfully transplanted with compatible marrow from a sister. Since then successful bone marrow transplantation from compatible donors has been carried out in patients with many other inborn errors of metabolism, mainly involving errors expressed in lymphocytes, phagocytes, erythrocytes and osteoclasts (Hobbs 1981).

The expectations and limitations of haematopoietic cell transplantation in the generalised inherited lysosomal storage disease α-mannosidosis (α-mannosidase deficiency) was described by Jolly *et al* (1976). A calf with mannosidosis, which was born as a twin with a normal calf of different sex, was shown to be chimeric in that it had a population of lymphocytes from both sexes, and serum α-mannosidase activity between normal and affected levels. There was a reduction in storage oligosaccharides in all tissues measured including brain and a marked reduction in vesiculation of visceral cells. However, the calf had neurological signs of advanced mannosidosis at 14 months when it was euthanased.

Studies have also been carried out on bovine generalised glycogenosis type II in Shorthorn cattle. It has been shown that purified enzyme is taken up by cultured skeletal muscle from affected animals and, after 48 hours in culture, the glycogen concentration in these muscle cells was comparable to the concentration observed in cultures from non-affected animals (Di Marco *et al* 1985). Intracellular levels of acidic α-glucosidase activity equivalent to

10% of the activity in normal muscle cultures appeared sufficient for the hydrolysis of stored lysosomal glycogen. These findings suggested that enzyme replacement therapy may be possible in this disease. Subsequently, Howell *et al* (1987) reported the use of multiple transplants of normal bovine amnion in bovine generalised glycogenosis type II. The morphological and biochemical changes associated with the disease were not significantly altered; however the treated animals lived as long, or longer than untreated animals maintained under similar conditions. Given the biochemical and morphological findings and the fact that it was difficult to rule out the effect of management factors, it was concluded that amnion transplantation could not be considered as a suitable method of enzyme replacement therapy for glycogenosis type II.

The efficacy of haematopoietic cell transplantation as a treatment for glycogenosis type II has also been investigated in twin cattle (Howell *et al* 1990). Over 95% of cattle twins have cojoint placentae and are blood chimeras. Three sets of such chimeras between normal and affected calves were studied. Increased activity of acidic α-glucosidase was detected in leucocytes, lymph nodes, spleen and liver from the affected calves, but the activity did not increase in muscle. Muscle lesions and clinical signs of glycogen storage disease developed.

In addition to the studies in cattle mentioned above, haematopoietic cell transplantation has also been described in canine α-fucosidosis (Taylor *et al* 1986, 1988, 1992, Taylor and Farrow 1988b); canine mucopolysaccharidosis type I (Shull *et al* 1987, 1988); murine globoid cell leukodystrophy (galactosylceramidase deficiency) (Hoogerbrugge *et al* 1988, Yeager *et al* 1984, 1991); murine mucopolysaccharidosis type VII (Birkenmeier *et al* 1991, Bou-Gharios *et al* 1992, Sands *et al* 1993); feline α-mannosidosis (Walkley *et al* 1994) and in feline mucopolysaccharidosis type VI (Gasper *et al* 1984). Donor-derived macrophages have been demonstrated in the central nervous system after bone marrow transplantation in mice affected with globoid cell leukodystrophy (Hoogerbrugge *et al* 1988). These cells have been reported to induce a partial reversal of the enzyme deficiency indicating that the blood-brain barrier is not necessarily an insurmountable obstacle to treatment of disease in the central nervous system.

Dogs affected with fucosidosis had increased levels of α-fucosidase enzyme activity in leukocytes, plasma, and neuronal and visceral tissues when bone marrow transplantation was carried out before the onset of clinical signs (Taylor *et al* 1986, 1988, 1992, Taylor and Farrow 1988b). Substantial enzyme replacement was shown in visceral organs and significant enzyme was demonstrated in the central nervous system, although this took up

to 6 months to establish. Reduced severity and slowed progression of clinical neurological disease was observed accompanied by a lesser degree of storage disease pathology, evident on histological evaluation. No obvious clinical benefit was evident in dogs engrafted for less than 6 months regardless of the age at which engraftment was performed, or in dogs with advanced disease. Similarly, dogs affected with mucopolysaccharidosis type I exhibited some degree of metabolic correction within the central nervous system after bone marrow transplantation at 5 months of age (Shull *et al* 1987, 1988).

The study in cats with α-mannosidosis also illustrated that a remarkable degree of correction can be achieved by bone marrow transplantation in a lysosomal storage disorder (Walkley *et al* 1994). In this study bone marrow transplantation was performed on three kittens with α-mannosidosis at 8, 10 and 12 weeks of age using phenotypically normal siblings as donors. End stage disease in untreated affected cats is usually reached by 6-8 months of age. The cats transplanted at 10 and 12 weeks were euthanased at 21 months and 11 months after transplantation respectively. In these two cats, skeletal abnormalities (patellar luxations and pelvic limb joint deformities) and mild symptoms of cerebellar dysfunction were reported. The cat transplanted at 8 weeks was still alive at 23 months of age with mild cerebellar signs but no skeletal deformities. Levels of activity of acidic α-mannosidase were determined in various tissues from the two cats which were euthanased. Enzyme activity varied remarkedly between tissues, i.e. from >100% of normal in CSF and bone marrow to only 3-6% of normal in kidney, but in all instances it was greater than that measured in an untreated animal with α-mannosidosis. These reports provide evidence that lysosomal hydrolases present in cells derived from donor bone marrow can gain access to the central nervous system and can significantly compensate for some genetic metabolic defects.

Bone marrow transplantation in murine mucopolysaccharidosis type VII has been shown to correct some biochemical and pathological abnormalities (Birkenmeier *et al* 1991, Bou-Gharios *et al* 1992, Sands *et al* 1993). However, bone marrow transplantation in the neonatal period in these and normal mice, had detrimental consequences (Bastedo *et al* 1994). In this study the regression in behaviour in transplanted normal mice and lack of improvement in transplanted mutants even though biochemical improvement was obtained, was attributed to the transplant procedure itself which involved exposure to radiation. This report highlights the harmful effects of marrow ablation particularly during the neonatal period. An alternative approach such as temporary enzyme replacement to prevent disease progression may be indicated during the newborn period until the transplant procedure can

be withstood. Neurological complications and neuropathological abnormalities resulting from haemorrhagic events and infections have also been reported in human patients after bone marrow transplantation (Mohrmann *et al* 1990). Patients requiring transplantation are predisposed to haemorrhage and infections both due to their disease-induced compromised state and due to the induced immunosuppression of the pre-transplant and post-transplant chemotherapeutic regimens and radiation therapy. Bone marrow transplantation is a procedure which has provided therapy for some life-threatening diseases but it is not without serious and broad-ranging side effects.

The efficacy of bone marrow transplantation in the treatment of metabolic storage diseases has been examined in children (Krivit and Shapiro 1991, 1994). Bone marrow transplantation is the only treatment available for many of these diseases and there are considerable risks and costs associated with the procedure. There are severe problems in assessing the response to this treatment in human patients in the absence of controls or the ability to accurately assess diseased organs. Bone marrow transplantation appears beneficial in late infantile and juvenile metachromatic leukodystrophy (arylsulphatase A deficiency); globoid cell leukodystrophy (galactocerebrosidase deficiency); adrenoleukodystrophy (very long-chain fatty acid accumulation); Hurler syndrome (α-L-iduronidase deficiency); Hunter syndrome (iduronate sulphatase deficiency) and Gaucher disease (glucocerebrosidase deficiency). Bone marrow transplantation had no beneficial outcome in Niemann-Pick disease (sphingomyelinase deficiency) or in glycogen storage disease type II (lysosomal acid α-glucosidase deficiency). Patients with other inherited storage diseases have also been transplanted, but the results have been inconclusive.

Foetal liver has been used as a source of haematopoietic stem cells and the precursors of hepatocytes to treat patients with inborn errors of metabolism (Touraine *et al* 1991). Pre-transplant conditioning was not required but moderate and prolonged immunosuppression was used. The clinical condition in some patients was improved after foetal liver cell transplantation but the procedure had to be repeated to maintain the benefit.

Human umbilical cord blood has been suggested as a possible source of stem cells for haematopoietic transplantation (Broxmeyer *et al* 1991). The use of umbilical cord blood avoids the invasive bone marrow harvest procedure and if cord blood could be harvested and stored routinely it would provide a valuable resource. However, only a limited number of stem cells are available from an umbilical cord blood sample and loss of some of these cells would be caused by cryopreservation. A child with Fanconi anaemia received a stem

cell transplant using umbilical cord blood from an HLA-matched sibling (Kohli-Kumar *et al* 1993). The engraftment was slow but it was comparable to other transplants in patients with Fanconi anaemia using HLA matched sibling bone marrow. There was no graft-versus-host disease.

It can only be concluded at this stage that, despite some obvious clinical successes in terms of disease modification, haemopoietic cell transplantation does not appear to be a panacea for all the inherited metabolic storage diseases. Many questions remain unanswered within this area and a true understanding of the principles and limitations involved remains to be fully elucidated.

Foetal Transplantation

Transplantation of haematopoietic stem cells as a treatment for children with genetic defects is limited by donor availability, graft rejection, the complications of immunosuppression and the occurrence of graft-versus-host disease. These problems can be overcome by transplanting normal haematopoietic stem cells from a preimmune foetal donor into an unrelated preimmune foetal recipient (Flake *et al* 1986, Harrison *et al* 1989, Zanjani *et al* 1991, Duncan *et al* 1992). Intraperitoneal foetal transfusion of blood for the treatment of rhesus sensitisation established this route as a possible method for haematopoietic transplantation (Liley 1963).

Immunocompetence develops early in all mammals of long gestation (Solomon 1971). There is thus a period of immunological tolerance early in foetal development when a foetus is incapable of rejecting transplanted cells. Transplantation in a preimmune foetus does not require recipient bone marrow conditioning regimes such as radiation or chemotherapy, or long term immunosuppression after grafting (Harrison *et al* 1989). A foetal donor is preferable as graft-versus-host disease does not develop (Zanjani *et al* 1991). Recipient compromisation due to the disease is minimised by foetal transplantation (Murnane *et al* 1991). This is of particular importance in neurodegenerative diseases. However, reliable prenatal diagnosis is a necessity and without cytoablative processes to enhance donor cell engraftment, only low levels of stable engraftment appear to occur.

Foetal therapy and gene therapy usually involve the use of haematopoietic stem cells. The majority of the mature haematopoietic cells are derived from only a few primitive stem cells.

Most haematopoietic stem cells are quiescent *in vivo* (Lemischka *et al* 1986). It is not practical to deplete the recipient's stem cells with radiotherapy and cytotoxic chemotherapy prior to foetal transplantation *in utero*. Transplanted stem cells are therefore in competition with endogenous stem cells. Engraftment of donor cells may occur but the proportion of active stem cells of donor origin giving rise to mature haematopoietic cells may be low in numbers relative to host cells. This was demonstrated by Pearce *et al* (1989) who attempted bone marrow transplantation by intraperitoneal injection of allogeneic cells into normal 60 day old female goat foetuses. The percentage of donor cell engraftment was 1-2% from the foetal donors but no chimerism was demonstrated in recipients of mature allogeneic bone marrow. Stable engraftment of myeloid and erythroid precursors in bone marrow and for blood lymphocytes has also been demonstrated in rhesus monkeys after *in utero* transplantation of foetal haematopoietic stem cells (Duncan *et al* 1992). The percentage of engrafted cells was 4-5% within the peripheral blood and bone marrow.

In many instances total replacement of the stem cell pool by donor cells may not be necessary but any procedure which can increase the proportion of donor cells may improve the therapeutic outcome. Possible manoeuvres to enhance the success of donor stem cell engraftment include increasing the dose of donor cells, introducing transplanted cells in a continuous steady fashion over an extended time course and using species-specific growth factors (Zanjani *et al* 1992).

Foetal stem cell transplantation minimises the immunological complications associated with cell transplantation and minimises the effects of the disease process. However, the practical limitations which include low engraftment levels, difficulties of *in utero* diagnosis in inherited diseases and ethical considerations which inhibit the use of liver stem cells from an aborted foetus, restrict the clinical applications of this procedure in human patients.

Gene Therapy

In recent years there have been significant advances in the knowledge of the molecular basis of inherited diseases and the genes responsible for many disorders have been cloned and characterised. Parallel advances have been made in developing techniques to introduce foreign gene sequences into cells. Gene therapy involves the insertion of an intact version of a gene into genetically compromised cells, thereby restoring their function. Genes can be transferred into germline or somatic cells. Ethical considerations preclude experimental

germline gene therapy in humans but genetic modification of a patient's somatic cells with the purpose of correcting severe genetic disorders has been deemed ethically acceptable (Anderson 1984, Walters 1986). Somatic gene therapy involves the insertion of the normal gene into target cells such that sufficient quantities of the gene product are produced in the cellular sites of pathology to correct the metabolic defect. Genes can be transferred to selected cells by virus vectors or by a number of physical or chemical procedures (Mulligan 1993).

Haematopoietic cells have been the main target of gene transfer studies. These cells have a wide distribution around the body and bone marrow transplantation has proved beneficial in some patients with genetic disorders. Most gene transfer studies have focused on using retroviruses as the vector but these viruses cannot infect non-dividing cells efficiently, can only accommodate approximately 10kb of foreign DNA and the fear remains that these viruses may be associated with the development of neoplasia. Other vectors including herpes simplex virus, parvovirus, and liposomes, as well as implantation of genetically modified fibroblasts have been proposed, and are under investigation. Parvoviruses have been reported to give high efficiency transduction into slow or noncycling immature and mature human haematopoietic progenitors, without requiring pre-incubation of cells in the presence of growth factors (Zhou *et al* 1994).

Retroviral-mediated gene transfer techniques have advanced rapidly during the past decade. Initial *in vitro* studies using recombinant retroviruses for gene transfer were encouraging, but early *in vivo* studies in mice were disappointing because of the transient and low levels of expression of the transferred gene (Lehn 1990). Retroviral technology for gene transfer *in vivo* relies on the use of "packaging cells". These cells express all viral proteins but do not produce infectious viruses themselves. The retroviral vector is transfected into packaging cells and its RNA is packaged by the viral proteins provided by the cells. These packaging cells allow the generation of helper-free replication-defective recombinant retroviruses capable only of "single hit" infection. Packaging cell lines have been constructed which provide a safe and efficient method for the transfer of genes in gene therapy protocols (Moen 1991, Einerhand and Valerio 1992). Studies have shown that long-term functional gene expression occurs after retroviral transfection of foetal liver *in utero* in rats (Clapp *et al* 1991), and after retroviral-mediated gene transfer to haematopoietic stem cells in mice (Holländer *et al* 1992).

Gene therapy has been attempted in β-glucuronidase deficient mice (mucopolysaccharidosis type VII) using microinjection of a human β-glucuronidase gene into nuclei of the deficient mouse zygotes (Kyle *et al* 1990) and by vector-mediated gene transfer to deficient mice (Moullier *et al* 1993, Wolfe *et al* 1992a, b). Transgenic mice expressed high levels of β-glucuronidase enzyme in tissues and were phenotypically normal after microinjection of a human β-glucuronidase gene into nuclei of deficient mouse zygotes (Kyle *et al* 1990).

Autologous implants of genetically modified skin fibroblasts on collagen lattices were implanted in the peritoneal cavity in mice affected with mucopolysaccharidosis type VII (Moullier *et al* 1993). These implants were reported to be stable for over 5 months. The macroscopic characteristics of the affected mice were not improved by the treatment but there was less histological evidence of lysosomal storage in the liver and spleen than that observed in un-implanted affected mice.

Retroviral vector-mediated gene transfer in cultured canine mucopolysaccharidosis type VII retinal pigment epithelial cells resulted in correction of the metabolic defect (Wolfe *et al* 1990). In retroviral vector-mediated transgenic mucopolysaccharidosis type VII mice, expression of low levels of β-glucuronidase over a long-term only partially corrected the disease (Wolfe *et al* 1992a). The results from these gene transfer studies involving cultured cells and animal models of human diseases are encouraging but problems remain to be overcome.

Retroviral-mediated foetal gene transfer has been successfully carried out in sheep (Kantoff *et al* 1989, Ekhterae *et al* 1990) but the gene transfer efficiency and expression levels were low. Hence the success of gene transfer would rely on a sufficient level of engraftment with gene transfer cells, or that low enzyme levels were sufficient to be corrective.

Nolta *et al* (1992) have demonstrated that retroviral vectors can efficiently transfer the glucocerebrosidase gene into long-lived haematopoietic progenitor cells from deficient Gaucher human bone marrow in culture. These cells reportedly expressed physiologically relevant levels of glucocerebrosidase enzyme activity.

Engraftment of haematopoietic stem cells can occur in completely untreated transplant recipients (Wu and Keating 1993). A technique has been described where canine haematopoietic stem cells in long-term marrow cultures were exposed to retrovirally carried genetic material on multiple occasions and triggered into entering the cell cycle

(Bienzle *et al* 1994). These activated cells continued normal programs of proliferation, differentiation and self-renewal *in vivo*. Their progeny were maintained at levels between 5 and 30% for up to 2 years in dogs without the requirement that endogenous haematopoiesis be suppressed through chemo or radiotherapy prior to adoptive transfer. This study indicated that haematopoietic stem cells in long term marrow culture may be ideal targets for gene therapy particularly in patients such as neonates where marrow-ablative conditioning is undesirable.

Gene therapy within the central nervous system can occur by colonisation of the brain by cells derived from retrovirus transduced haematopoietic stem cells with a similar effect to bone marrow transplantation. Alternatively DNA sequences can be introduced into the central nervous system by *(a)* injection of a recombinant virus vector, *(b)* by transplanting cells that have been infected with a virus vector or *(c)* transplanting cells which have been genetically modified by a number of physical or chemical procedures.

Herpesvirus vector-mediated gene transfer was carried out into post-mitotic neurons in mice affected by mucopolysaccharidosis type VII (Wolfe *et al* 1992b). Cells expressing β-glucuronidase enzyme activity were present in the mice brains for up to 4 months post-innoculation. This study demonstrated the potential of this approach for long-term expression of foreign genes in the central nervous system but too few cells were corrected to alter the disease phenotype in these mice.

Transplantation of cells within the central nervous system is a possible therapeutic approach for certain neurodegenerative disorders. Human patients with severe Parkinson disease have received grafts of foetal central nervous system tissue (Lindvall *et al* 1990, Spencer *et al* 1992). Functional improvement has been reported in some patients but this procedure is still considered experimental (Doering 1994). The results from foetal tissue grafts have raised the possibility that similar, or more successful treatment, may be provided by genetically modifying some cells from a patient and directly implanting these modified cells into the brain. This approach has been taken by Doering and Chang (1991) who have transfected rat fibroblasts by calcium phosphate precipitation with a plasmid that encoded human growth hormone and the neomycin resistance genes. These fibroblasts were grafted into the brains of other rats. The gene product was successfully expressed in the brain and also entered the systemic circulation but the length of time of expression was limited. Further investigation is needed in this area to improve the stability of gene expression, the

longevity of the implant and to determine the reaction of the host immune system to the new cells and their gene products.

Organ, tissue and cell transplantation has been one of the major therapeutic advances of our time. This review discussed this methodology particularly in relation to lysosomal storage diseases. There has been some success in treating some of these diseases but there are many equivocal results as well as high risks and high costs associated with this therapy. It is anticipated that molecular biology through gene therapy will provide superior and eventually less expensive treatment. Gene therapy became a clinically relevant procedure with the treatment of the first human patient (Culliton 1990). Many inherent problems remain unsolved in this new field but the clinical applications can be expected to expand rapidly.

Chapter Three

General Materials and Methods

Animals

Sheep with ceroid-lipofuscinosis were bred in a flock maintained for this purpose by mating 7-8 month old homozygous affected ram lambs with heterozygous ewes. To maintain genetic variation and to increase flock numbers, homozygous ram lambs have on occasion been outbred to normal Romney ewes. Resultant obligate heterozygous ewe lambs were retained in the breeding flock. Control tissues were obtained from age, and usually sex, matched New Zealand Romney sheep. All sheep were maintained on pasture at Massey University under standard New Zealand husbandry conditions.

Preclinical Diagnosis of Lambs with Ceroid-lipofuscinosis

Diagnosis of ceroid-lipofuscinosis in lambs before they were clinically affected was carried out at 2 $^{1}/_{2}$ - 3 months of age by histopathology of brain biopsies obtained by the method of Dickson *et al* (1989), with minor modifications (see chapter four). In brief this procedure was carried out under general anaesthesia and involved obtaining a 14 G needle biopsy core of grey matter from the cerebral cortex. This tissue was fixed in 10% neutral buffered formalin, embedded in paraffin wax and stained with Sudan black. When examined under a light microscope, black granular material was observed in the neuronal cytoplasm in biopsy sections from affected lambs. Preclinical diagnosis of ceroid-lipofuscinosis made by brain biopsy was later confirmed either by development of characteristic clinical signs, evidence of brain atrophy and/or histopathology.

Euthanasia and Treatment of Tissues

For most purposes and unless otherwise stated, sheep affected with ceroid-lipofuscinosis and normal control animals were euthanased at 12-24 months of age. This was carried out by intravenous sodium pentobarbitone, or sodium thiopentone anaesthesia followed by

exsanguination by severance of carotid arteries and jugular veins when in the anaethetised state. Tissues were obtained within minutes of euthanasia and placed in chilled 0.1 M phosphate buffered saline pH 7.2, frozen at either -20 or -70 degreesCelsius, or fixed in 10% neutral buffered formalin for light microscopy or 3% glutaraldehyde in 0.1 M phosphate buffered saline pH 7.2 for electron microscopy.

Isolation of Storage Bodies

Weighed amounts of pancreas or liver were homogenised in 10 volumes of distilled water for 60 seconds in a Sorvall Omnimixer (Ivan Sorvall Inc.). The homogenate was sonicated for 3 minutes at approximately 14 microns amplitude using a MSE Soniprep 150 ultrasonic disintegrator then filtered through glass wool and pelleted by centrifugation at 1,400g_{max} for 30 minutes at 4°C in a Sorvall general laboratory bench centrifuge (GLC-1), or a MSE Mistral 6L centrifuge. The resultant supernatant was discarded, the pellet resuspended in deionised water and then centrifuged at 12,000g_{max} for 20 minutes at 4°C in a Sorvall RC5 centrifuge (Dupont). The supernatant was again discarded and any intermediate layer material present on top of the pellet was gently washed off with deionised water. This preparation was divided for further processing for electron microscopy, polyacrylamide gel electrophoresis (PAGE), fluorescence studies, or resuspended in distilled water and stored at -20°C until required.

Extraction of Proteolipid from Storage Bodies

The pellet of storage body material suspended in a small volume of deionised water was pipetted slowly into 40 volumes of chloroform/methanol (2:1 v/v) containing 100mM ammonium acetate in a conical flask on an automatic magnetic stirrer. The resulting solution was filtered through Whatman no. 1 filter paper into a separating funnel, where 0.2 volumes of deionised water was added. The flask was gently swirled then either left to stand while the contents separated into two phases or the bottom phase was run through phase separating paper (Whatman 1PS). If the bottom phase appeared cloudy a few drops of methanol was added. The sample was then dried in a rotovap (Buchi Rotavapor-R) at 35°C to approximately one fifth volume. Ten volumes of diethyl ether was added and the flask sealed and allowed to stand in an ice-salt bath for 2 hours while the proteolipid precipitated.

Proteolipids were harvested by centrifugation at 1,400g_{max} for 5 minutes at 4°C in a Sorvall general laboratory centrifuge (GLC 1). The proteolipid pellet was redissolved in chloroform/ methanol (2:1 v/v) with 100 mM ammonium acetate. After taking a 200 µl sample for amino acid analysis, the solution was sealed in glass vials and stored in a dark room.

Fixation and Preparation of Tissues for Electron Microscopy

Representative samples of storage body preparations and cubes of tissue <1 mm^3 obtained as soon as possible after death were fixed in 3% glutaraldehyde in 0.1 M phosphate buffered saline pH 7.2. These samples were post-fixed for 1 hour in 1% osmium tetroxide, dehydrated in graded alcohols and propylene oxide then embedded in epoxy resin. Thin sections (60-100 nm) were stained for 6 minutes with saturated uranyl acetate, rinsed in distilled water, then stained for a further 6 minutes with saturated lead citrate, rinsed again, then left to air dry. Sections were examined in a Phillips 200 transmission electron microscope.

Histopathological Methods

Slices of tissue 5-7 mm thick were fixed in 10% neutral buffered formalin and processed into paraffin wax. Whole brains were fixed in excess of 20 volumes of 10% neutral buffered formalin, then 5-7 mm thick slices were processed into paraffin wax. Histological sections of these tissues were stained with Haematoxylin and Eosin (H & E), Periodic Acid Schiff (PAS) (Culling 1974), Luxol Fast Blue (LFB), Sudan Black (Culling *et al* 1985) and a section left unstained for fluorescence microscopy.

Fluorescence Microscopy

Fluorescence of unstained sections was examined using an Olympus BH-2 microscope (Olympus, Japan), barrier filter 530 nm, excitation filter 440 nm.

Amino Acid Analysis

Samples were hydrolysed *in vacuo* in 6 M hydrochloric acid containing 0.1% phenol at 110°C for 24 hours. The hydrolysates were dried *in vacuo* over NaOH pellets and quantitative amino acid analysis was carried out on a Beckman 119L amino acid analyzer. Protein concentrations were calculated from this data.

Lithium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (LDS-PAGE)

Soluble proteins were separated by LDS-PAGE by the method of Laemmli (1970) on 15% and 20% polyacrylamide gels with a bisacrylamide to acrylamide ratio of 1:29 w/w and with 10% w/w sucrose added to the main gel. Fresh and frozen protein samples were dissolved in 1% LDS and added to a boiled solution containing 12% glycerol and 6 μg/ml bromophenol blue solution. Apparent molecular weights of proteins were determined by comparison of their migration rates with those of molecular weight standards (Sigma kit no. MW-SDS 70L).

Silver Staining of Polyacrylamide Gels

After electrophoresis (and after electroblotting if this was carried out), proteins were fixed in the gel by immersion for 1 hour in 12% w/v trichloroacetic acid. The gels were then washed for 1 hour in 40% methanol containing 10% acetic acid, washed twice for 30 minutes each wash in 10% ethanol containing 5% acetic acid and finally washed for 10 minutes in an oxidising solution containing 3.4 mM potassium dichromate and 3.2 mM nitric acid. After removal of the oxidising agent from the gels by washing in deionised water, they were stained for 10 minutes in 12 mM silver nitrate. Gels were then rinsed for 2 minutes with deionised water and developed by successive additions of a solution containing 0.28 M sodium carbonate and 6.33 mM formaldehyde. Development was continued until the desired staining intensity was reached and then curtailed by washing the gels for 5 minutes in 5% (v/v) acetic acid. Addition of the silver nitrate solution and subsequent colour development steps were carried out under a photographic safe light (Kodak, Wratten series filter OB). The gels were destained by incubation in a solution of 30 mM potassium ferricyanide and 65 mM sodium thiosulphate (Farmer's reducer). This reducing agent was

completely removed by multiple washes in deionised water and the gels were stained again in 12 mM silver nitrate and developed as before.

Quantitative Extraction of Subunit c from Tissues

Two grams of tissue (pancreas or liver) was homogenised in 30 ml chloroform/methanol/ ammonium acetate (2:1:100 mM) for 1 minute. This homogenate was transferred into a flask and combined with the homogeniser rinsings which used a further 10 ml chloroform/ methanol/ammonium acetate (2:1:100 mM). After incubation for 1 hour at room temperature on an orbital shaker (Lab-line Instruments Inc., Melrose Park, ILL, USA) at 150 RPM this homogenate suspension was filtered. The residue was resuspended in 40 ml chloroform/ methanol/ammonium acetate (2:1:100 mM) and incubated again as above, then filtered and combined with the initial filtrate. Serial dilutions were made in triplicate ranging from 1:1 to 1:512. Aliquots of 3 µl were dotted onto wet polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA), these membranes were dried and stored at 4°C.

Chemiluminescent Detection of Extracted Subunit c

Proteins on the PVDF membranes (from above) were detected using the Biorad Immun-lite II chemiluminescent protein detection system (Biorad Laboratories, Hercules, CA, USA). All incubations were carried out at room temperature. PVDF membranes were made wet in methanol for 3 seconds then washed twice in Tris-buffered saline (TBS) containing 200 mM Tris and 5 M sodium chloride with pH 7.5, for 10 minutes each wash. This was followed by incubation for 30 minutes in 5% non-fat dried milk in TBS and a further two washes for 10 minutes each in TBS containing 0.05% Tween-20 (TTBS). Primary antibody (anti-subunit c -RabA see chapter seven) was applied after dilution to 1:3000 in 1% non-fat dried milk in TTBS for 3 hours. The membranes were then washed three times for 10 minutes each wash and incubated for 1 hour in secondary antibody, consisting of a 1:300 dilution of goat anti-rabbit-alkaline phosphatase conjugate in 1% non-fat dried milk in TTBS. Again the membranes were washed three times for 10 minutes in TTBS, incubated in enhancer solution, rinsed quickly in TTBS, incubated in substrate solution, drained and sealed in plastic. These PVDF blots were exposed to hyperfilm-ECL (Amersham International Inc., Buckinghamshire, UK) for 3, 5 and 8 minutes. The film was developed in a Kodak X-Omat

automatic processor (Eastman Kodak Co., Rochester, NY, USA). Densitometry measurements were carried out by Dr. W.T. Jordan at Victoria University, Wellington, New Zealand.

Special Methods

Special methods relating to particular experiments are included in the appropriate chapters.

Chapter Four

Diagnosis of Ceroid-lipofuscinosis in Live Sheep

Introduction

Clinical signs of ceroid-lipofuscinosis in sheep develop slowly and progressively after 7 months of age. They include delayed response to the presence of an observer; a depressed eye preservation (menace) response; holding their heads high when travelling and tilting their heads to move from one visual field to another; grazing alone; lagging behind the flock when moved and becoming difficult to control with a sheep dog (Jolly *et al* 1980, Mayhew *et al* 1985).

Early diagnosis of the disease is necessary so that animals can be used for breeding and consequently they can be available for experimental purposes at an early age. Diagnosis of the disease in lambs is carried out routinely by histopathology of brain biopsies prior to the onset of clinical signs. This is necessary so that the flock of sheep can be maintained by mating homozygous affected 7-9 month old ram lambs with heterozygous ewes.

This section records *(a)* the results of brain biopsies carried out for the early diagnosis of ceroid-lipofuscinosis and *(b)* the development of a semi-objective means of assessing the onset and progression of clinical disease ("clinical staging").

Materials and Methods

Brain Biopsies

Brain biopsies were carried out according to the method of Dickson *et al* (1989) with minor modifications. Prior to surgery, lambs were given a subcutaneous injection of 900,000 units of procaine penicillin and 900,000 units of benethamine penicillin (Propen LA, Glaxo Animal Health N.Z. Ltd). In 1989-1990 anaesthetic induction was by intravenous 5% sodium thiopentone (Intraval Sodium, May and Baker Ltd., Dagengam, England) at a dose of 10-15 mg/kg liveweight. From 1991 anaesthetic induction was by a mixture of 0.5 mg/

kg diazepam (Pamlin, Parnell Laboratories, New Zealand limited) and 5 mg/kg ketamine (Parnell Laboratories, New Zealand limited) given intravenously to effect. Lambs were then intubated, placed in sternal recumbancy and anaesthesia was maintained using a mixture of up to 2% halothane and 30 ml/kg oxygen on a rebreathing circuit. An area over either the left or the right frontal bone was clipped and shaved from just behind the orbit to in front of the ear prior to routine surgical skin disinfection and draping.

An area approximately 1-2 cm lateral to the midline and 1 cm caudal to a line joining the lateral canthi of the eyes was surgically prepared and a 1 cm incision made through skin and underlying fascia. A 2.0 mm hole was drilled through the frontal bone with a sterile dental air drill, with care taken to avoid puncturing the dura mater. A 14 G needle cut down to 4-6 mm with a non-bevelled but sharpened end, attached to a 10 ml syringe was inserted through the hole. On contact with the dura mater the syringe plunger was withdrawn to the 5 ml mark and the needle was gently bored through the dura and 2-3 mm into the cerebral cortex by a to and fro rotatory motion. A core of cortical tissue was removed by withdrawing the needle while maintaining negative pressure. This was expelled immediately into 10% neutral buffered formalin. If a clearly visible core of tissue was not obtained the procedure was repeated once only. The skin incision was closed with a single interrupted suture of 0.5 metric monofilament nylon. The cerebral cortex tissue sample was processed into paraffin wax and 3-4 μm sections were cut and stained with Sudan black.

Clinical Staging of Ceroid-lipofuscinosis

The methodology for clinically staging ceroid-lipofuscinosis was based on the clinical description of the ovine form of the disease by Mayhew *et al* (1985). The neurological status of normal lambs and lambs with ceroid-lipofuscinosis was assessed at monthly intervals over a 7 month period, from 5 months of age. This was carried out "blind" by two veterinarians (including the author) who reached a consensus on scoring. Initially a full neurological examination following the method of Brewer (1983) was carried out on each sheep. Many of the parameters examined, such as patella reflexes and crossed extensor reflexors, were found to be unhelpful in the diagnosis of ceroid-lipofuscinosis. These aspects of the neurological examination were abandoned and a modified procedure evaluating 5 different parameters was adopted. In the modified procedure, head carriage and postural traits were assessed while the sheep were herded from the paddock into a darkened shed. The sheep were then handled individually and the "menace" and "blink to bright light"

responses were assessed. The final part of the examination consisted of observing the sheep negotiate a simple maze and assessing the ease with which they traversed the obstacles (sight and cognitive faculties). The maze involved the sheep being released into a race, changing direction to find their way obliquely across a pen, going through an open gateway, up a step, changing direction again and going down a step to join other sheep. For each category the assessment for each sheep was given an arbitrary maximum of 10 with a range of 0-10. A zero score was given for a totally absent response, while delayed or otherwise abnormal responses were graded between these values. The scores at each examination were summed and expressed as a "neurological score".

Results

Brain Biopsies

Over a 5 year period 252 sheep were subjected to brain biopsy (Table 4-1). Lambs could be clearly diagnosed by histopathological examination as affected with ceroid-lipofuscinosis, or normal, depending on whether or not a proportion of neurons contained several Sudan black stained inclusions. In 1990 two deaths occurred. One of these lambs was euthanased after 3 hours of assisted breathing following anaesthesia as it could not breath unaided. Postmortem examination showed this lamb had "enzootic pneumonia" which had not been detected by clinical examination prior to anaesthesia. The other lamb died during recovery after uneventful surgery and anaesthesia. No abnormalities were evident at post mortem examination. All other lambs (250) recovered rapidly from anaesthesia, and there were no losses or complications directly attributable to the surgery. Skin wounds healed by first intention as observed when the sutures were removed at 7-10 days after surgery.

Samples were obtained which enabled diagnosis in 247 of the 252 sheep biopsied (98%). Two errors in diagnosis were made, one false positive and one false negative. These were related and occurred due to a clerical error in the handling of specimens. Samples which were unsuitable for diagnosis consisted solely of white matter without grey matter, meninges, a blood clot, bone fragments, or various combinations of these.

Table 4-1: *The Results of Brain Biopsies in Lambs for the Diagnosis of Ceroid-lipofuscinosis by Histopathology.*[+]

Year	Number of biopsies total	Biopsy number positive	Biopsy number negative	Clinical* number positive	Clinical* number negative	No sample	Errors		Deaths
							False positive	False negative	
1989	44	21	23	20	22	0	1#	1#	0
1990	68	24	42	24	42	2	0	0	2
1991	53	28	25	28	25	0	0	0	0
1992	42	17	24	17	24	1	0	0	0
1993	45	18	25	18	25	2	0	0	0
Total	252	108	139	107	138	5	1	1	2

\+ All biopsies were carried out by a surgical team including and organised by the author.

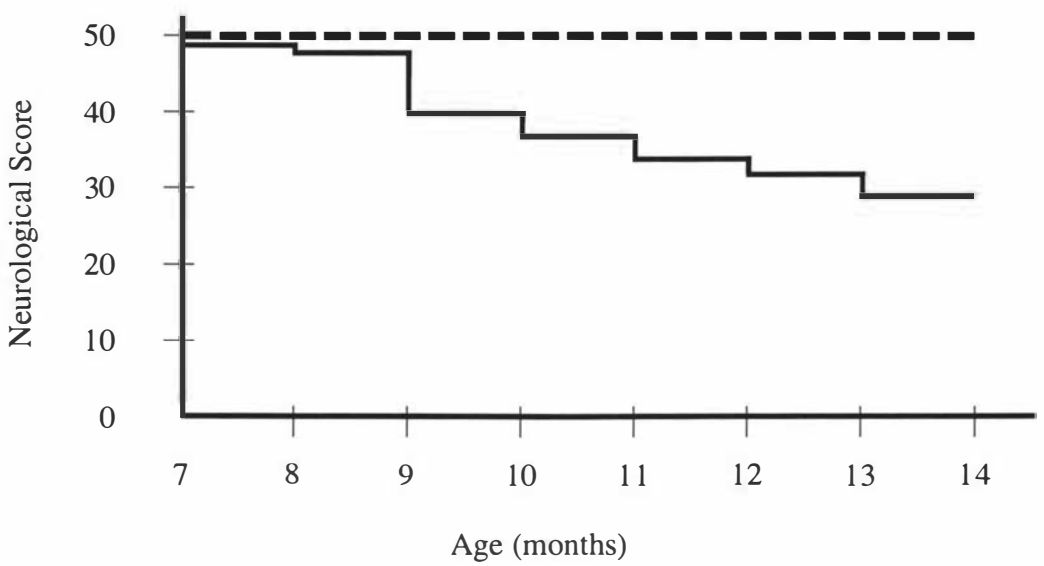
* Eventually developed clinical disease and/or confirmed by further histopathological examination.

\# Error due to specimen handling.

Clinical Staging of Ceroid-lipofuscinosis

Objective assessment of early neurological manifestations was difficult but as the disease progressed the clinical signs became increasingly pronounced (Figure 4-1).

Figure 4-1: *Clinical Staging of the Development of Ceroid-lipofuscinosis in Sheep.*



Key

Phenotypically normal lambs *(total = 11).*

Lambs affected with ceroid-lipofuscinosis *(total = 5).*

Discussion

Brain biopsy followed by histological staining with Sudan black has proved to be a safe and reliable technique for diagnosis of ceroid-lipofuscinosis in 2 $^{1}/_{2}$ month old lambs. Affected lambs can be clearly distinguished from normal lambs with the only errors in the procedure being attributable to human error during handling of specimens. Over a 5 year period 252 lambs were biopsied, and two deaths occurred. One of these deaths was attributed to "enzootic pneumonia". This is a low grade chronic non-progressive pneumonia in lambs endemic in most flocks (Blood *et al* 1983). Cases may be difficult to detect and it is a factor that may compromise anaesthesia. Anaesthesia poses the greatest risk factor and rigorous attention to detail is necessary. Intubation is considered essential. In the three years succeeding the deaths the anaesthetic agent was changed from sodium thiopentone to a diazepam/ketamine mixture and clinical examination procedures prior to anaesthetic have become more rigorous. Since then all lambs have recovered uneventfully without any complications attributable to the surgery or anaesthesia.

No sample of cerebral cortex suitable for diagnosis was obtained in 5 out of 252 lambs biopsied (2%). Marked individual variation in bone thickness of the skull was seen in the lambs, with the bigger ram lambs in particular often having comparatively thick bone. This may have contributed to the poor samples obtained in some cases. One lamb was electively euthanased 10 days after brain biopsy. At post mortem examination minimal scarring of the meninges and a slight depression in the cerebral cortex were evident at the biopsy site.

Clinical staging of the progression of ceroid-lipofuscinosis, especially in the early stages of the disease was difficult. This is due to the insidious onset of the disease and slow progression of clinical signs. However with time, a clear distinction can be made between phenotypically normal and ceroid-lipofuscinosis affected sheep.

In the flock a higher proportion of the lambs diagnosed by brain biopsy are phenotypically normal compared with those diagnosed as affected with ceroid-lipofuscinosis. It could be expected from mating homozygous affected ram lambs with heterozygous ewes that equal numbers of both normal and affected lambs would be born. The higher proportion of normal lambs can probably be attributed to the existence of some non-heterozygous ewes within the flock. Heterozygous animals appear phenotypically normal and there is no diagnostic method available to confirm the genetic carrier status of these animals.

Summary

A brain biopsy technique has proved to be safe and reliable for the diagnosis of ceroid-lipofuscinosis in 2 $^{1}/_{2}$ month old lambs. Diagnosis by a clinical staging procedure was difficult in the early stages of the disease but with time a clear distinction can be made between phenotypically normal and ceroid-lipofuscinosis affected sheep.

Chapter Five

Haematopoietic Cell Transplantation in Ceroid-lipofuscinosis Affected Foetal Lambs

Introduction

Haematopoietic cell transplantation has proved a successful treatment for some genetic diseases (see chapter two). These results have encouraged surgeons to carry out haematopoietic cell transplantation in other diseases where children are severely affected by inherited metabolic defects and where current conventional management and treatment offered to these patients is ineffective. For many inherited metabolic diseases the risks and costs associated with haematopoietic cell transplantation compare favourably with current methods of treatment. However, early intervention is essential as the secondary effects of disease such as the loss of neurons may be irreversible.

The ultimate therapy for inborn errors of metabolism would be gene therapy. The normal gene would be inserted into some somatic cells from a patient and these cells would be reimplanted to provide an endogenous source of gene product. Haematopoietic stem cells could be suitable carriers for therapeutic genes as they have the ability to differentiate into a variety of progeny cells including erythroid, thromboid, myeloid, macrophages and lymphoid cells. Macrophages derived from haematopoietic stem cell transplantation circulate widely reaching most tissues including the central nervous system (Hoogerbrugge *et al* 1988). The effectiveness of haematopoietic cell transplantation, where macrophages are a supplier of corrective enzymes, is hence a prerequisite for attempting gene therapy.

The complete lack of any effective treatment for ceroid-lipofuscinosis and the relentlessly progressive tragic nature of the disease leads desperate parents to consider treatment options such as bone marrow transplantation where there is any possibility the outcome may be favourably altered (Parental statement, 5th International Conference on ceroid-lipofuscinosis, Newark, USA, 1994). However, given the high risk and ethical requirements of trying treatment options in children, animal models have a role to play in evaluating the effectiveness of new therapeutic regimes. This study was initiated in conjunction with a

group of bone marrow transplant therapists from the USA, with the objective of investigating the use of haematopoietic cell transplantation in foetal lambs affected with ceroid-lipofuscinosis. This would allow intervention as early in the disease process as practical. Foetuses at 58-60 days gestation are not yet immunologically competent (Solomon 1971) thus tissues from normal donors can be used without having to match HLA tissue antigens.

Materials and Methods

Animals

The recipients were South Hampshire foetuses of haemoglobin BB bloodtype at 58-60 days of gestation. These were the result of mating BB bloodtype ewes heterozygous for the ceroid-lipofuscinosis genotype with homozygous ceroid-lipofuscinosis affected 6 month old ram lambs of BB bloodtype. As such these recipient lambs would be either homozygous affected genotype or heterozygous clinically normal phenotype. The latter were to act as controls. The donors were normal Romney foetuses at 45-48 days of gestation of AA bloodtype. These were the result of mating normal Romney ewes with normal Romney rams of AA bloodtype. To ensure the foetuses were the correct ages, the oestrus cycles of all the ewes were synchronised by the use of progesterone impregnated intravaginal devices (CIDRs, Ciba-Geigy, New Zealand).

Surgery

All surgical procedures were performed using standard aseptic surgical techniques. Prior to surgery all sheep were starved for 24 hours and water was withheld for 12 hours. The donor ewes were anaethetised using 35-40 ml 5% sodium thiopentone via the jugular vein and the uterus was exteriorised, ligated and removed via a ventral midline incision. The donor ewes were then euthanased using sodium pentobarbitone.

The foetus/foetuses were removed from the uterus and their sex noted by examination of their external genitalia. The liver was removed and homogenised in a Potter-Elmingehem homogeniser in Iscoves Modified Dulbecco medium (IMDM) supplemented with up to 10% foetal calf serum and heparin. An aliquot was taken for cell counting using a haemocytocrit and the number of cells (hepatocytes and haematopoietic) in the total

preparation was calculated. Livers from both male and female foetuses were homogenised together. The liver homogenate was centrifuged and the pellet resuspended at the desired concentration in IMDM.

The recipient ewes were given 60 mg of progesterone parenterally 16 hours prior to surgery to ensure uterine relaxation and help prevent spontaneous abortion (Pearson and Mellor 1977). Immediately prior to surgery they were given 4 ml Streptopen antibiotic (Pitman-Moore, New Zealand Limited) subcutaneously. This antibiotic cover was continued for the following 3 days after surgery. Anaesthesia was induced in the recipient ewes using sodium thiopentone; they were then intubated and anaesthesia maintained by halothane and oxygen. After routine skin preparation and draping, a ventral midline incision was made through the abdominal wall. The uterus was exteriorised and the foetus located and held gently while the uterus was superficially incised and blunt dissected to the chorionic membrane. Hepatic donor cells (10^{10} cells/kg estimated foetal body weight) were injected through the chorionic membrane into the peritoneal cavity of the foetal lamb. The abdominal wall was closed and skin sutured using standard surgical techniques. The sheep were hospitalised for 7 days following surgery. At 10-13 days post-surgery the stitches were removed. All recipient ewes recovered uneventfully.

Note: Surgery was performed by two surgical teams over a 4 day period. One of these teams included the author who was also responsible for the overall logistical arrangements of the procedure and for care of the animals. The uterine surgery was performed by Professor D. Mellor. The preparation and peritoneal injection of foetal liver cell suspensions was carried out by Dr I. Zanjani.

Engraftment of Donor Cells

Blood and bone marrow samples were taken at 2, 9 and 15 months after birth. Donor cell engraftment was monitored by isoelectric focusing and chromatography of haemoglobin on carboxymethylcellulose columns and by karyotype analysis of peripheral blood lymphocytes and bone marrow progenitor-derived erythroid and myeloid haematopoietic colonies (Zanjani *et al* 1991).

Note: Isoelectric focusing, chromatography and karyotype analysis were carried out by Dr I. Zanjani.

Diagnosis of Disease Status

A needle biopsy of cerebral cortex was obtained from transplanted and non-transplanted lambs at 2.5 months of age by the method of Dickson *et al* (1989) and fixed in 10% neutral buffered formalin. Microscopic examination of Sudan black stained paraffin sections allowed diagnosis of affected or non-affected individuals (see chapter three).

Neurological Examination

The neurological status of each sheep was assessed at monthly intervals over a 7 month period from 5 months of age as described in chapter three. This was carried out by two veterinarians, including the author, who reached a consensus on scoring for each of 5 different characteristics. Initially head carriage and postural traits were assessed while the sheep were herded from the paddock into a darkened shed. Following this the sheep were handled individually and the "menace" and "blink to bright light" responses were assessed. The final part of the examination consisted of observing the ease with which the sheep traversed a few obstacles. This involved the sheep being released in a race, changing direction to find their way obliquely across a pen, through an open gateway, up a step, changing direction again and going down a step to join other sheep. Head carriage and postural responses were again assessed during this phase. For each category the assessment for each sheep was given an arbitrary maximum of 10 with a range of 0 - 10. A zero score was given for a totally absent response, while delayed or otherwise abnormal responses were graded between these values. The scores at each examination were summed and expressed as a "neurological score". This scoring system was subjective, and as the disease process was progressive, the worst overall score, previous or current, was used.

Euthanasia and Gross Pathology

Lambs were euthanased with sodium thiopentone anaesthesia followed by exsanguination. A postmortem examination was carried out on all sheep involved in this study and brain weights recorded.

Fixation and Preparation of Tissues for Microscopy

Tissue samples approximately 5-7 mm thick from pancreas (right lobe), liver (mid cranial right lobe), kidney (cranial third of right kidney), and cardiac muscle (tip of left ventricle) were taken from each sheep during post mortem examination and fixed in 10% neutral buffered formalin. Brains were fixed whole. Blocks from each tissue and cerebral cortex (level with the caudal corpus callosum), cerebellum (mid saggital section) and brain stem (caudal to fourth ventricle) were embedded in paraffin wax and microtome sections (7μm) were stained with periodic acid schiff (PAS), luxol fast blue (LFB), Sudan black (SB), haematoxylin and eosin (H&E). One section was deparaffinised but left unstained for fluorescence microscopy.

Scoring of Histopathological Sections

Staining intensity and the amount of storage material stained by various stains was arbitrarily graded by consensus of two pathologists including the author. An increasing intensity scale with values based on the photomicrographs of Jolly *et al.* (1989) was used where "0" equated to no evidence of storage material, and "5" equated to the amount of storage material evident in sections from severely affected animals at a late stage in the disease course.

Retinal Lesion Assessment Scores

At post mortem examination an eye was removed from each animal. An incision was made posterior to the corneo-scleral junction and the anterior segment removed. The vitreous humor was extracted and the posterior segment was fixed in 10% neutral buffered formalin. After fixation a retinal section was taken by dividing the posterior section in an anterior-posterior direction through the optic disk. This sample was embedded in paraffin wax by routine methodology and sectioned for histological staining and microscopic examination. The H&E stained retinal sections were scored "blind" by the two pathologists (including the author) on a consensus basis. An assessment of retinal atrophy was made on the basis of the number of cells in the outer nuclear layer and of the proportion of cones to rods. An arbitrary scoring system was used ranging from 0 to 5, where "0" was a normal retina, while "5" would have been for the severe changes seen in advanced ovine ceroid-lipofuscinosis

(Jolly *et al* 1982, Graydon and Jolly 1984). In instances where retinal sections exhibited regional differences a score was given based on the overall impression of the atrophic changes.

Quantitative Assessment of Subunit c in Tissues

Quantitative extraction of subunit c from tissues and chemiluminescent detection of this extracted subunit c was carried out as described in chapter three.

Results

Foeti from 28 sheep received in-utero haematopoietic cell transplantation. Donor cell engraftment was monitored at 2, 9 and 14 months after birth, and identified nine lambs with transplanted cells. Of these four were shown to have ceroid-lipofuscinosis by histopathology of brain biopsies and five were unaffected (Table 5-1).

Table 5-1. *Donor Cell Engraftment (%) in Lambs After Intraperitoneal Foetal Haematopoietic Stem Cell Transplantation at 58-60 Days Gestation.**

Cell Type	Diagnosis	Age of Lambs (months)		
		2	9	14
Erythroid	OCL	4.68 ± 0.31	6.54 ± 0.60	6.17 ± 0.51
	N	5.22 ± 0.32	5.81 ± 0.54	5.76 ± 0.58
Myeloid	OCL	7.23 ± 0.63	14.94 ± 1.60	12.97 ± 1.39
	N	8.43 ± 0.70	11.55 ± 1.40	11.38 ± 0.98
Lymphoid	OCL	6.85 ± 0.21	9.57 ± 0.42	9.93 ± 0.53
	N	7.50 ± 0.36	8.86 ± 0.35	9.42 ± 0.44

OCL Ceroid-lipofuscinosis affected lambs *(total = 4).*

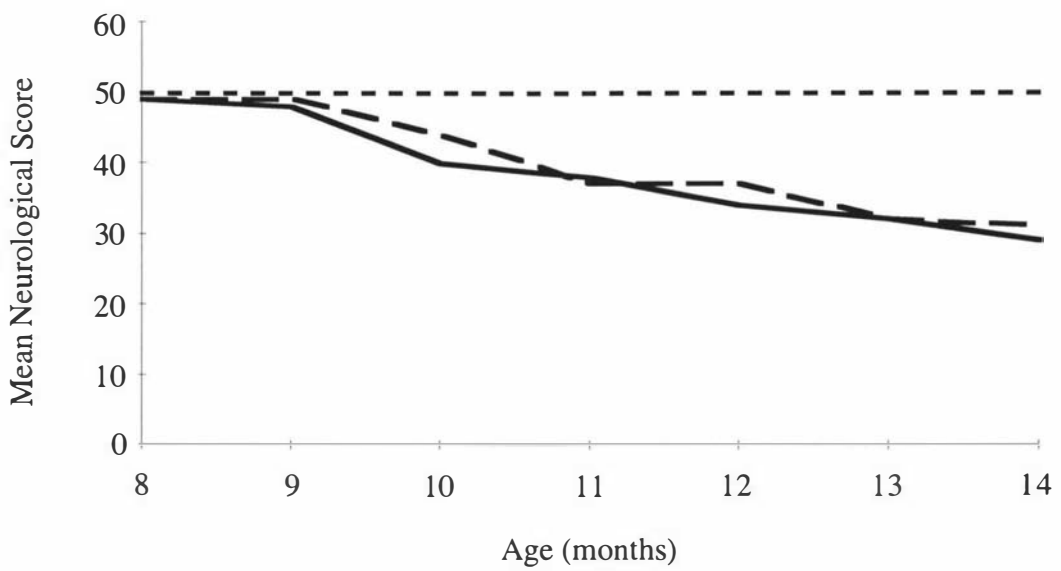
* N Phenotypically normal lambs *(total = 5).*

± Standard error.

Neurological Assessment

No difference was observed in the clinical progression of neurological disease between the transplanted and untransplanted sheep affected with ceroid-lipofuscinosis (Figure 5-1).

Figure 5-1: *Staging of the Clinical Progression of Disease Symptoms in Transplanted (total = 4) and Untransplanted (total = 5) Sheep Affected with Ceroid-lipofuscinosis.*



Key

- - - - - - Transplanted and non-transplanted phenotypically normal lambs *(total = 11)*.

— — — Transplanted lambs affected with ceroid-lipofuscinosis *(total = 4)*.

——— Non-transplanted lambs affected with ceroid-lipofuscinosis *(total = 5)*.

Gross Pathology

Variable degrees of brain atrophy were observed in affected sheep at autopsy (Table 5-2). There was no significant difference between the weights of brains from transplanted and non-transplanted affected lambs. In contrast there was a significant difference (T-test $p<0.01$) between the brain weights of all affected lambs when compared to normal controls.

Table 5-2. *The Effect of Haematopoietic Cell Transplantation in Foetal Lambs with Ceroid-lipofuscinosis as Evaluated by the Severity of Lesions Noted in Various Tissues.**

Parameter	**Transplanted** OCL *total = 4*	**Non-transplanted** OCL *total = 5*	**Normal**[d] *total = 11*
Brain weight[a]	66.0 ± 2.8 g	65.0 ± 5.5 g	88.0 ± 9.0 g
Retinal atrophy[b]	2.6 ± 1.1	1.3 ± 0.8	0.0
Pancreas[c]	2.2 ± 0.4	2.3 ± 0.5	0.0
Liver Kupffer cells[c]	3.2 ± 0.8	3.5 ± 0.5	0.9 ± 0.8
Liver hepatocytes[c]	3.2 ± 1.3	3.8 ± 0.4	0.0
Kidney[c]	3.6 ± 0.5	3.7 ± 0.5	1.8 ± 0.4
Cerebal cortex[c]	3.0 ± 0.0	3.2 ± 0.4	0.0
Cerebellum[c]	1.8 ± 0.2	2.0 ± 0.3	0.0

* OCL Ceroid-lipofuscinosis affected lambs.

± Standard deviation.

[a] Transplanted and non-transplanted vs. normal brain weights; significant $P < 0.01$.

[b] Mean score of the degree of retinal atrophy in histological sections.

[c] Mean intensity score of the amount of storage material in Sudan black stained histological sections.

[d] Transplanted and non-transplanted phenotypically normal lambs.

Histopathology

Characteristic storage cytosomes in the brain of ceroid-lipofuscinosis affected sheep were noted with H&E, SB, LFB and PAS staining and also by fluorescence microscopy. In other tissues the LFB stain showed no staining, and PAS method showed variable staining. In all tissues (normal and affected) fluorescence was observed from red blood cells, and fluorescent material was present in Kupffer cells in the liver and kidney parenchymal cells. Assessments of tissue pigment storage were carried out using SB stained sections (Table 5-2) but there were no observed differences between the transplanted and untransplanted affected sheep groups. A laminar zone deficient in neurons was observed in sections of cerebral cortex taken through the occipital pole in all affected lambs. This was not observed in the normal controls. In all cases the retinas of normal sheep were scored 0 but there was considerable variation in the severity of the retinal changes between the different affected sheep (Table 5-2).

Quantitative Detection of Subunit c by Enhanced Chemiluminescence

Pancreatic tissue samples were too small and not of sufficient quality (fibrous tissue, blood and fat were attached) to allow quantitative subunit c extraction. Quantitative extraction was successfully carried out from liver samples. Densitometry measurements of reaction product in the various serial dilutions were compared. The liver from normal sheep contained an average of 468 μg subunit c per gram of tissue, the liver from sheep affected with ceroid-lipofuscinosis contained an average of 2,591 μg subunit c per gram of tissue and the liver from sheep affected with ceroid-lipofuscinosis which received haematopoietic cell transplantation contained an average of 1,765 μg subunit c per gram of tissue. There was no significant difference between the amount of subunit c extracted from transplanted affected sheep compared with the amount extracted from untransplanted affected sheep (T-test $p > 0.05$). There was however a significant difference (T-test $p < 0.01$) between the amount of subunit c extracted from both these groups of affected sheep and the quantity extracted from phenotypically normal sheep.

Discussion

This study attempted to incorporate the optimal conditions for successful treatment of ceroid-lipofuscinosis by haematopoietic stem cell transplantation except that ablative processes to enhance donor cell engraftment were not possible under the conditions of foetal transplantation. In this trial foetal lambs were transplanted at 58-60 days gestation (gestation in sheep is 145 days), a time prior to the onset of overt disease or immunological competence (Solomon 1971). The donor cells were from normal non-heterozygote foetal lambs and would be expected to eventually express the full complement of normal enzymes. Foetal livers were chosen as the source of cells as this is the primary organ of haematopoiesis at this gestational age in foetal lambs.

An average of 9% engraftment of donor cells was achieved. However no clinical, pathological or biochemical benefit could be demonstrated in affected lambs. In recording the data, only that for brain weight and quantitative subunit c extraction from liver, were fully objective. The other semi-objective data were scored by consensus of two clinicians/ pathologists under "blind" experimental conditions. For this reason levels of significance have not been stated although standard deviations are included for pathological data to indicate the range of variation recorded. Comparison of the retinal sections from different sheep was particularly difficult as there were sampling errors due to inconsistency both in the plane of section and of the exact retinal region examined and in some cases the different retinal layers had separated and parts were missing in the sections.

Specific cell layers within the cerebral cortex are difficult to identify in non-primates. However, in the cerebral cortex of all affected lambs there was a laminar zone deficient in neurons in occipital pole sections. This finding of selective necrosis of certain neurons has also been reported in the juvenile and late infantile forms of disease (Zeman *et al* 1970, Braak and Goebel 1978, 1979, Jolly 1995) and in affected sheep (Jolly *et al* 1989). It has been postulated that this loss of cells implies a toxicity inherent in the disease process, rather than cell death due to overloading of the cells with storage bodies *per se* (Jolly 1995).

There are two likely reasons for the failure of haematopoietic stem cell transplantation to affect the ceroid-lipofuscinosis disease process. Firstly this may be a disease that is not amenable to treatment by metabolic collaboration with the transplanted cells. Secondly the level of donor cell transplantation (approximately 9%) may be too low to produce any discernible benefit to nervous or visceral systems.

In normal tissues the levels of any enzyme present exceeds the basic minimum required for any substrate metabolism. The progression of any metabolic storage disease will be influenced by the presence and amount of residual enzyme activity. It is reasonable to assume "that the more profound the enzyme deficiency, the more severe the disorder and conversely the higher the residual activity of the mutant enzyme the greater the protection against disease" (Neufeld 1991). A model of this concept (Conzelmann and Sandhoff 1983/84) proposes a critical threshold of activity above which the enzyme is capable of keeping up with the substrate metabolism and below which it cannot, so that accumulation occurs. The kinetics are such that at the critical threshold of enzyme activity, even quite small amounts of additional enzyme could markedly affect the rate of substrate accumulation and hence progression of disease. Therefore 9% transplanted cells might be able to influence the course of disease in some way. However, under the conditions of this experiment such transplantation failed to influence the disease progression in the recipient lambs. Nevertheless the feasibility of foetal transplantation of haematopoietic stem cells was demonstrated. Methods of improving the ratio of donor to recipient cells in tissues need to be developed. One example of this which has been described involved using modified media to enhance haematopoietic stem cell colony growth (Zanjani *et al* 1990). This technique enabled enhancement of donor cell engraftment from original levels between 4-15% to 12-16%, but still further enhancement of engraftment beyond these levels would be desirable. The engraftment level of 9% in this study compared favourably to the engraftment levels gained in an equivalent study in goats reported by Pearce *et al* (1989) (see chapter two).

The nature of the storage disease has an influential effect on the likely outcome of enzyme or haematopoietic cell transplantation. The metabolic defect in ceroid-lipofuscinosis remains undefined but at least in the late infantile form it is unlikely to be a cathepsin deficiency (Ezaki *et al* 1993, 1995). Storage material may also be complexed in such a tertiary structure within cells that it is resistant to the effect of endogenous enzymes or exogenous enzyme replacement. Subunit c of mitochondrial ATP synthase has a propensity for self aggregation due to its hydrophobicity and it is possible that this property may be involved in the pathogenesis of its accumulation (Jolly 1995).

Allogenic bone marrow transplantation has also been carried out in English Setter dogs affected with ceroid-lipofuscinosis at 4 $^{1}/_{2}$ months of age (Deeg *et al* 1989, 1990). This treatment was unsuccessful in altering the course of the disease in the four dogs transplanted but cells from heterozygous donors were used which may not have expressed optimal levels of enzyme activity and the transplantation may have been too late in the disease course. The

experiences of Taylor *et al* (1986, 1988, 1992) and Taylor and Farrow (1988b) in bone marrow transplantation of dogs with fucosidosis, showed that the age of engraftment was important with best results occurring with early engraftment. Central nervous system enzyme replacement was a gradual process taking up to 6 months to achieve substantial levels compared with less than 2 months in other tissues.

Bone marrow transplantation has been attempted in a clinically unaffected yet pathologically affected sibling of a patient with late infantile Batten disease (Lake *et al* 1995). The effect of the transplantation was monitored for 9 months until the donor marrow became displaced by the host cells. No removal of storage material could be demonstrated but the patient passed the age at which his sibling developed symptoms by 3 months. This apparent postponement of symptoms could be the effect of natural variation in the onset of disease, or more encouragingly, could be due to transplantation preventing further build-up of storage material.

Lake *et al* (1995) carried out co-culture experiments using lymphocytes from normal subjects cultured with those from a clinically unaffected yet pathologically affected sibling of a patient. Pathognomic curvilinear bodies were found in these co-cultured lymphocytes up to and including the 6th day, indicating the bodies remain in the cells in culture. Messer *et al* (1995) describe the fusion of homozygous *mnd* mouse embryos (a model for Batten disease) with normal embryos. In these embryos neurons filled with storage material could be seen adjacent to normal cells implying that metabolic collaboration did not occur.

The tight junctions between endothelial cells which form the blood-brain barrier and prevent plasma proteins entering the brain are present from very early in foetal development (Bradbury 1979). However, various studies using a variety of test compounds (including inulin, sucrose, mannitol, adenine, arginine, choline, tryptophan, pyruvate and lactate) have shown that the rate of entry of these substances into the brain is much higher in the foetus and up until just after birth, than in older animals. It is theoretically possible that this increased permeability of the blood-brain barrier may also facilitate optimal colonisation of the central nervous system by transplanted normal donor cells. Even if this is not the case, studies on bone marrow transplantation performed in both animals and children several weeks or more after birth have shown transplantation to be clearly beneficial to central nervous system disease for example, in α-mannosidosis cats transplanted at 8, 10 and 12 weeks after birth (Walkley *et al* 1994), see chapter two. Hence consideration of the blood-brain barrier as an impediment to the correction of central nervous system disease after bone

marrow transplantation may be overstated as neuronal enzyme replacement has been achieved by the invasion of cells of haematogenous origin (Taylor *et al* 1986, 1988, 1992, Shull *et al* 1987, 1988, Hoogerbrugge *et al* 1988, Birkenmeier *et al* 1991, Yeager *et al* 1991, Krivit and Shapiro 1991, Walkey *et al* 1994). Other studies also provide evidence that microglial cells within the brain are derived from bone marrow precursors (Perry *et al* 1985, Hickey and Kimura 1988, Hickey *et al* 1992, Unger *et al* 1993).

Extensive research effort is currently being expended in the area of gene therapy. The idea of gene transfer is worthy but the current severe technical limitations of early ceroid-lipofuscinosis diagnosis, gene transfer and engraftment preclude successful practical application of this technique at this stage. It remains necessary to prove that treatment by exogenous enzymes, or treatment by some other process using transplanted cells is beneficial in Batten disease before effort is expended in the area of gene transfer.

Given that conventional medicine currently fails totally in the treatment of patients with ceroid-lipofuscinosis, it is extremely disappointing that there is very little evidence thus far to provide any support for the efficacy of bone marrow transplantation in this disease. Appropriate tissue culture models of this group of diseases are now available (Ezaki *et al* 1993, 1995, Bennett *et al* 1993, Dunn *et al* 1994, Lake *et al* 1995). Further investigation on cell culture mechanisms of metabolic collaboration in this disease are warranted before further transplantation studies are carried out.

Summary

Haematopoietic cell transplantation was carried out in foetal lambs affected with ceroid-lipofuscinosis at 58 - 60 days of gestation. Cells from homozygous normal foetal donors were used. An average of 9% engraftment of donor cells was achieved but no clinical or pathological benefit was demonstrated. Two possible reasons why this procedure was unsuccessful were proposed; firstly, the level of engraftment may have been too low and secondly, ceroid-lipofuscinosis may not be a disease which is amenable to treatment by metabolic collaboration.

Chapter Six

Fluorescence of Storage Bodies and Reconstitution of Storage Bodies from Isolated Components

Introduction

Historically the ceroid-lipofuscinoses have been grouped with the lipidoses on the basis of their histological staining properties which have implied a lipid-based pathogenesis of these diseases. The stored material in ceroid-lipofuscinosis also shows yellow autofluorescence under blue light and this observation has lead to the assumption that some intrinsic fluorophore is present. Intensive research and much speculation has been expended trying to determine the nature of this fluorophore, as clarification of this issue has often been considered central to the elucidation of the pathogenesis of the ceroid-lipofuscinoses. Chio and Tappel (1969a, b) reported that Schiff base polymers with the same fluorescence as lipofuscin were created by the reaction of malonaldehyde, a product of peroxidation of polyunsaturated fatty acids with phospholipids or the amino groups of proteins. This schema appeared to be directly applicable to Batten disease and consequently abnormal lipid peroxidation was proposed as being central to the pathogenesis of this group of diseases (Zeman and Dyken 1969, Zeman 1974, Zeman 1976). As a direct result of this hypothesis, biochemical studies in Batten disease have focused almost exclusively on lipids until comparatively recently. This has been discussed in detail in chapter one.

Studies on the emission spectra of the stored material in late infantile human, English setter dog and South Hampshire sheep forms of Batten disease showed emission in the yellow-orange region (520-540 nm) and not in the blue region (460-480 nm) expected for the particular Schiff base polymers (Eldred *et al* 1982, Dowson *et al* 1982, Katz *et al* 1988) that result from lipid peroxidation. Therefore it was considered unlikely that the autofluorescent granules which accumulate in tissues as a result of ceroid-lipofuscinosis are products of lipid peroxidation. Alternatively, if the disease-related fluorophors are the result of lipid peroxidation, they must be generated via mechanisms substantially different from those which occur *in vitro*. Studies have shown that peroxidation artefacts can be produced during

organic solvent extractions (Katz *et al* 1988, Kikugawa *et al* 1994). Processes which produced this peroxidation resulted in the generation of blue fluorescence from tissues showing yellow fluorescent lipofuscin *in situ.*

Palmer *et al* (1986a) extracted small quantities of a number of weakly fluorescent lipids from sheep liver storage bodies using chloroform-methanol and reported that the mass of residual insoluble material remaining after lipid extraction was not fluorescent. It was concluded that these fluorescent lipids were carotenoids resulting from vitamin A and chlorophyll breakdown and not related to the disease.

This chapter explores the phenomenon of storage cytosome fluorescence and describes the reconstitution of liposomes from normal phospholipids with subunit c isolated from ovine storage bodies.

Materials and Methods

Fluorescence of Storage Bodies in Suspension

Storage bodies isolated from ovine, canine and human late infantile forms of ceroid-lipofuscinosis (see chapter three) were suspended in a water:glycerol mixture (1:1). These suspensions were then examined under an Olympus BH-2 microscope with an Olympus BH2-RFL reflected light fluorescence attachment (Olympus Optical Co, Ltd, Tokyo, Japan) using a 440 nm excitation filter and a 530 nm barrier filter.

Absorbance Spectra of Dissolved Storage Bodies

Ovine pancreatic and liver storage bodies (see general materials and methods, chapter three) were dissolved in 1% lithium dodecyl sulphate (LDS) and in chloroform/methanol/ ammonium acetate (2:1:100mM). Extracted subunit c obtained after ether precipitation was also dissolved in chloroform/methanol/ammonium acetate (2:1:100mM). Absorbance spectra of these solutions were measured in a double beam ratio reading SP8-400 UV-Vis spectrophotometer (Pye Unicam Ltd, York Street, Cambridge, England). Fluorescence spectroscopy was carried out in an Aminco SPF-500 spectrofluorometer (American

Instrument Company, Silverspring, Ohio, USA). Quinine sulphate at a concentration of 0.1 μg/ml in 0.05 M sulphuric acid was used as the fluorescence standard.

Phospholipid Extraction

Phospholipids were extracted from egg yolk and normal sheep liver. This was carried out by homogenising 10 g of egg yolk or liver in 200 ml of chloroform/methanol (2:1). The resulting suspension was filtered into a separating funnel and 40 ml 0.74% potassium chloride was added. These separating funnel contents were vigorously mixed then allowed to stand until the two phases separated. The lower phase was collected and evapourated to dryness in a rotary evaporator, then resuspended in 5 ml of chloroform. Phospholipids were precipitated by the addition of 50 ml of acetone at 4°C for 1 hour, followed by centrifugation at 1,400g_{max} for 5 minutes at 4°C in a Sorvall general laboratory bench centrifuge (GLC-1). After this centrifugation the supernatant was discarded and the precipitate rinsed with cold acetone, centrifuged as above and again the supernatant was discarded. The precipitate was dried with nitrogen gas and then resuspended in 10 ml of chloroform. A measured aliquot of this solution was then taken so that the concentration of phospholipids in chloroform could be determined from the dry weight.

The Manufacture of Liposomes

Concentrations of subunit c in chloroform/methanol/ammonium acetate (2:1:100mM) were determined by quantitative amino acid analyses (see chapter three). Known ratios (by weight) of either egg yolk or liver phospholipids and subunit c proteolipid were combined and the absorbance spectra of these solutions measured. To make liposomes, solutions of phospholipids and proteolipid were combined in small lidded flasks. The solvent was evapourated to dryness under nitrogen at 35°C and approximately 1 ml 0.67% potassium chloride solution was added to each flask for every 20 mg phospholipid and proteolipid present. The flasks were sealed and shaken on a Lab-line orbital shaker (Lab-line Instruments Inc, Melrose Park, Illinois, USA) at 37°C for 12 hours then sonicated at 10 μ for 10 seconds. Aliquots were taken for fluorescence microscopy and electron microscopy. The latter were fixed in 3% glutataldehyde in 0.1 M phosphate buffer and processed as recorded in chapter three.

Results

Fluorescence of Storage Bodies, Dehydrated Protein Aggregates and Phospholipids in Suspension

Isolated liver and pancreas storage bodies from ovine, canine and human late infantile forms of ceroid-lipofuscinosis exhibited sparkling yellow-green fluorescence when suspended in a water:glycerol mixture (1:1) and viewed in a fluorescence microscope (Figures 6-1 and 6-2). Protein aggregates of bovine serum albumin and casein also exhibited sparkling yellow-green fluorescence when suspended in a water:glycerol mixture and viewed in the same way (Figure 6-3). Similar suspensions of phospholipids did not show any fluorescence.

Absorbance Spectra of Dissolved Storage Bodies *in Vitro*

Ovine pancreatic and liver storage bodies dissolved completely in 1% LDS. The UV-visible absorbance spectra of these solutions was unremarkable, there being an absorbance maximum at 275 nm and a small shoulder at 412 nm (Figure 6-4). Similar spectra were obtained from the total chloroform/methanol soluble components of the storage bodies (lipids plus the proteolipid) and the ether precipitated subunit c redissolved in chloroform/methanol. When fluorescence spectroscopy of these solutions was attempted, high concentrations were required to get any signal at all, even at the most sensitive settings of the instrument. A concentration of 1.38 mg/ml, redissolved liver storage body proteolipid gave a weak spectrum with an emission maximum of 500 nm and a smaller peak at 680 nm when using an excitation maximum of 400 nm. These emission and excitation maxima peak heights were equalled by those of a 0.1 μg/ml solution of quinine sulphate (excitation maximum 350 nm, emission maximum 450 nm). Dilution of the samples resulted in a proportionate drop in signal strength, eliminating the possibility that internal absorption of the emitted light caused the lack of observed fluorescence.

Liposomes Containing Subunit c

Storage body-like structures were reconstituted when chloroform/methanol solutions of phospholipids and proteolipid were mixed, the solvent evaporated and the resultant film sonicated in aqueous buffer. This reconstitution resulted in similar structures over a wide

Figure 6-1: *Fluorescence of Storage Body Aggregates Isolated from the Pancreas of a Human Patient with Late Infantile Batten Disease Suspended in Glycerol/Water* x 400. *Fluorescence Microscopy, Excitation Filter 440nm with 530nm Barrier Filter.*

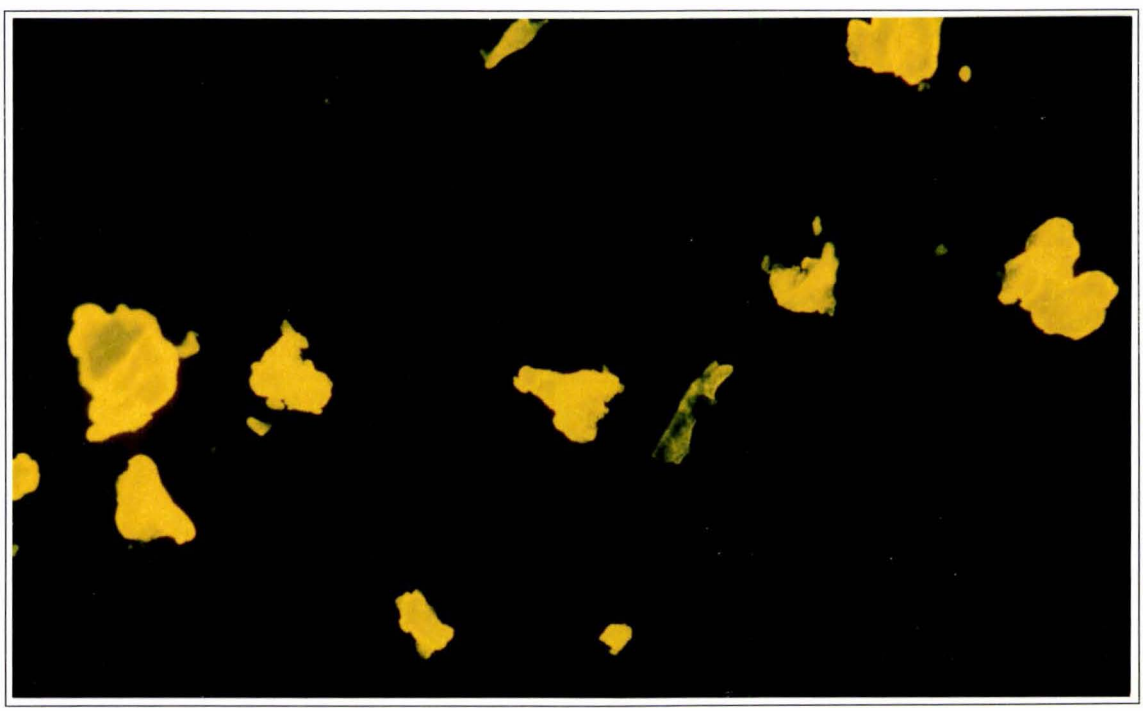


Figure 6-2: *Fluorescence of Storage Body Aggregates Isolated from the Pancreas of a Sheep Affected with Ceroid-lipofuscinosis Suspended in Glycerol/Water* x 400. *Fluorescence Microscopy, Excitation Filter 440nm with 530nm Barrier Filter.*

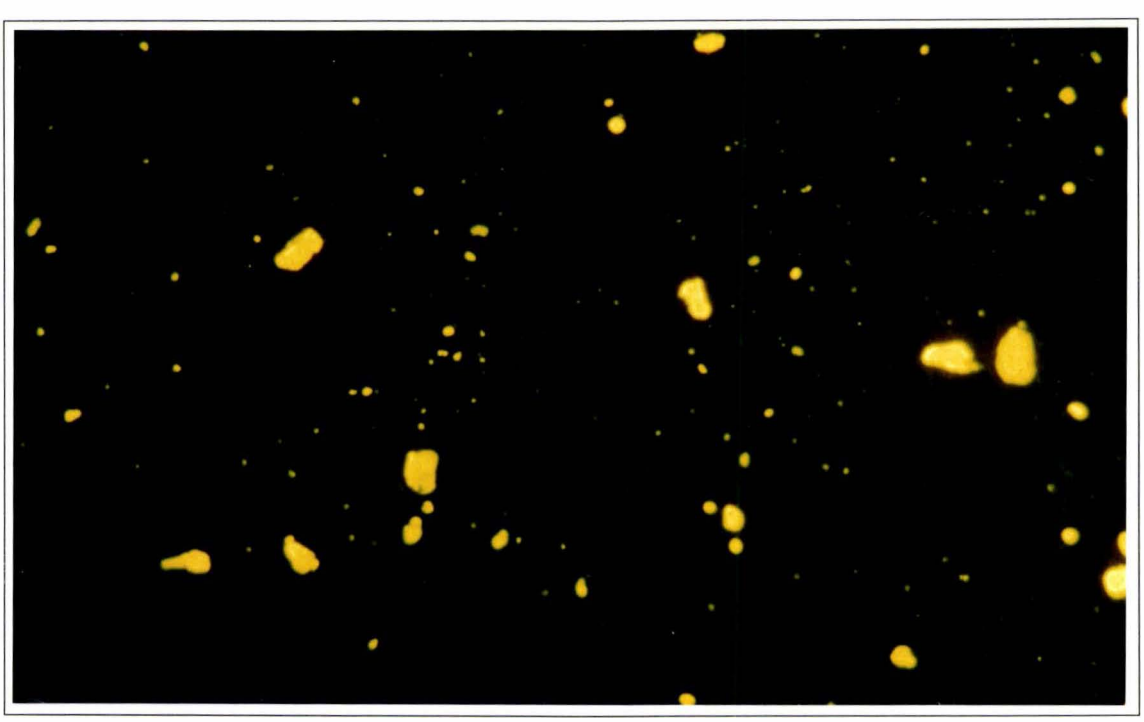


Figure 6-3: *Fluorescence of Bovine Serum Albumin Aggregates Suspended in Glycerol/Water* x 100.
Fluorescence Microscopy, Excitation Filter 440nm with 530nm Barrier Filter.

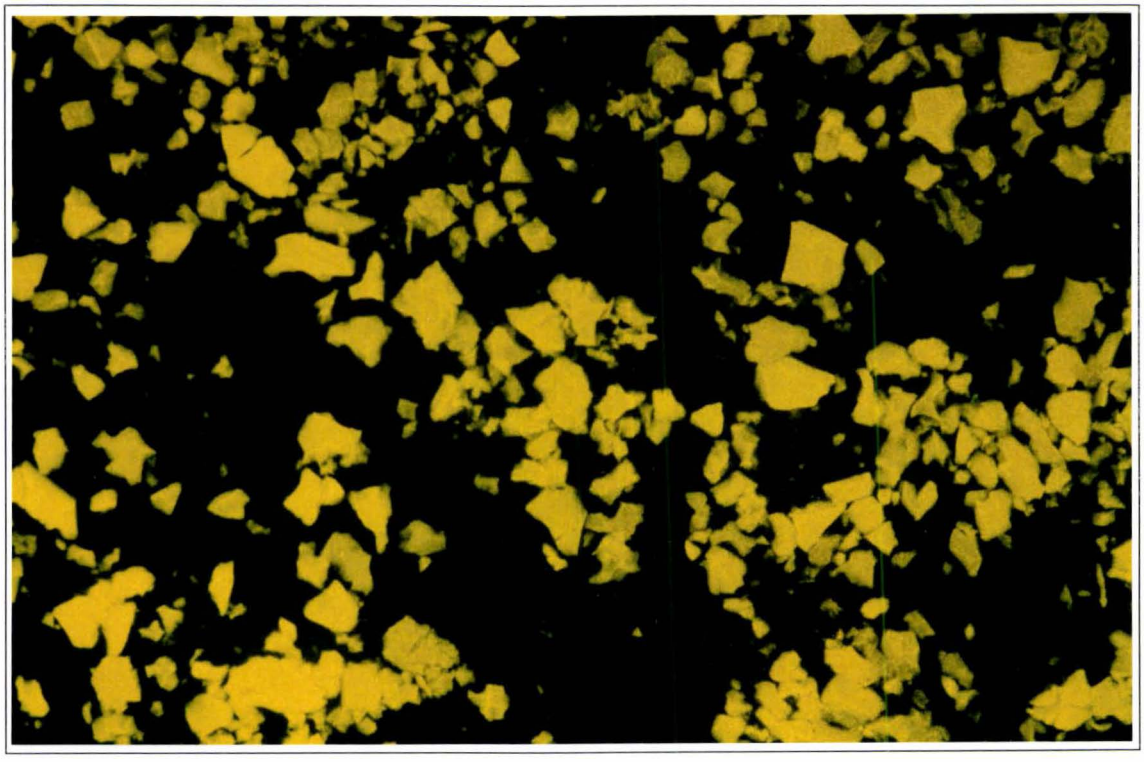
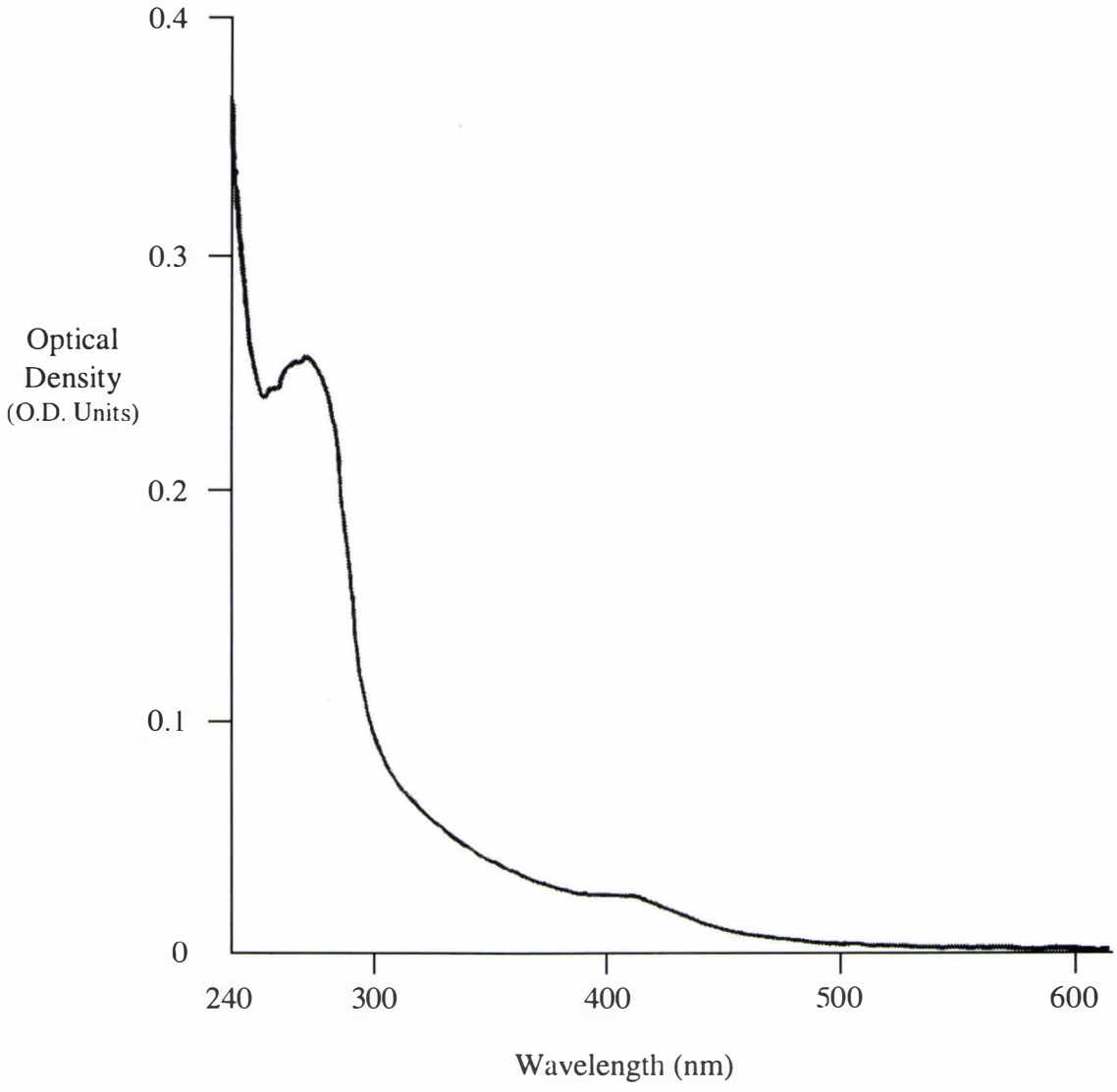


Figure 6-4: *UV-visible Absorbance Spectrum of Proteolipid Extracted from Sheep Liver Storage Bodies in Chloroform/Methanol.*



range of proteolipid to phospholipid ratios (2:1-1:20 w/w), the excess phospholipid remaining as long strands of phospholipid bilayer. The reconstituted bodies exhibited somewhat similar multilamellar ultrastructure to that shown by isolated storage bodies (Figures 6-5 and 6-6) whereas the proteolipid alone was quite amorphous (Figure 6-7). Areas similar to those often referred to as lipid droplets were also observed under some conditions (Figure 6-8). The components of the reconstituted bodies were non-fluorescent when examined in solution, singly or combined, under a fluorescence microscope or in a spectrophotometer. However, the reconstituted bodies exhibited similar sparkling fluorescence to the storage bodies when viewed in a fluorescence microscope.

Discussion

Fluorescence is the name given to the phenomenon whereby a body emits light during the absorption and excitation by a primary light source. The emitted light is always of a longer wavelength than the primary excitation light. Some substances fluoresce in the visible region when excited with light of an appropriate wavelength without being specially treated, stained or labelled. In a histological context this phenomenon is called autofluorescence to distinguish it from the fluorescence seen after using histological stains that exhibit fluorescence. Light is absorbed by molecules into certain well defined quantised energy states. The accompanying transition of an atomic or molecular orbital from a lower to a higher energy state, which is accomplished by the absorption of the radiant energy, is the basis of all methods of absorption spectroscopy. The electrons in an atomic or a molecular orbital may subsequently descend from the higher energy state back to a lower one, a transition that is usually accompanied by the emission of radiant energy. This transition is the basis of all methods of emission spectroscopy. Many molecules may absorb energy but do not fluoresce because all the energy absorbed is rapidly lost by collisional deactivation and released as heat (Guilbault 1990). Fluorescence cannot occur without light absorption but any portion of the spectrum where absorption occurs can produce fluorescence. For any single molecule the fluorescence peak will be at the same wavelength regardless of the excitation wavelength used but the intensity of emission will vary with the relative strength of the absorption.

Fluorescence in ceroid-lipofuscinosis is not a simple phenomenon. These, and previous studies (Palmer *et al* 1986a, b, Katz *et al* 1988) have shown it cannot be attributed to the existence of a single identifiable fluorophore, with identifiable molecular properties

Figure 6-5: *Electron Micrograph of Liposomes Made From Liver Phospholipids and Subunit c Proteolipid (20:1)* x 33,500.

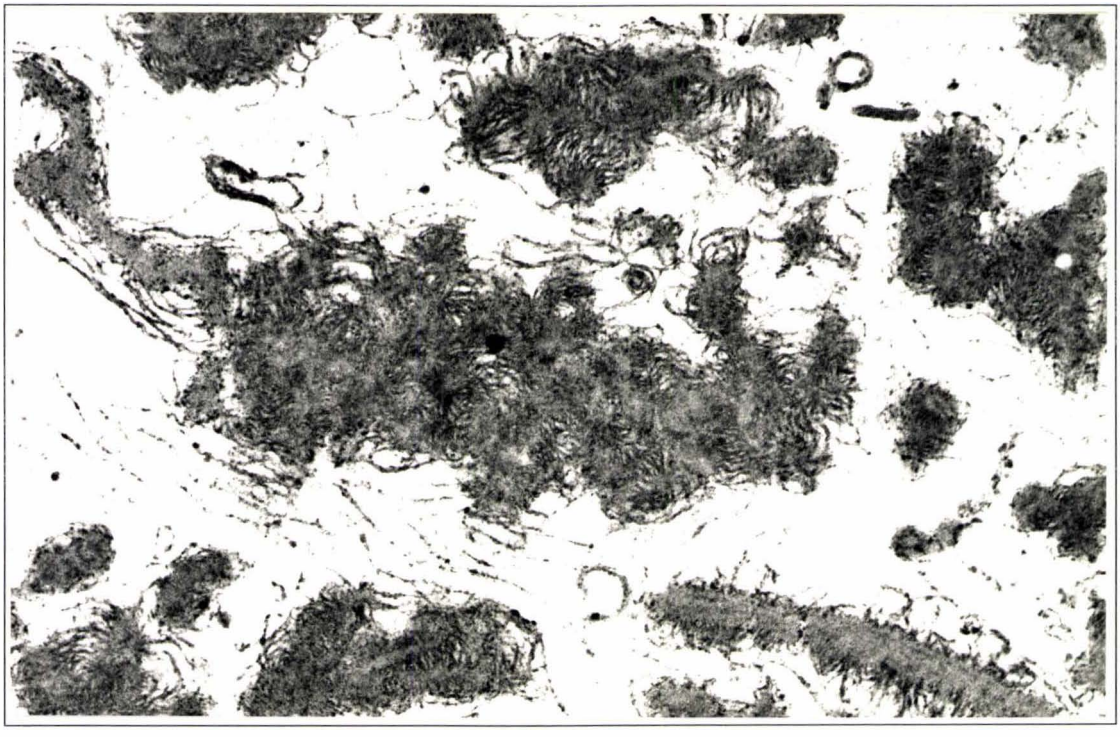


Figure 6-6: *Electron Micrograph of Liposomes Made From Egg Phospholipids and Subunit c Proteolipid (20:1)* x 12,500.

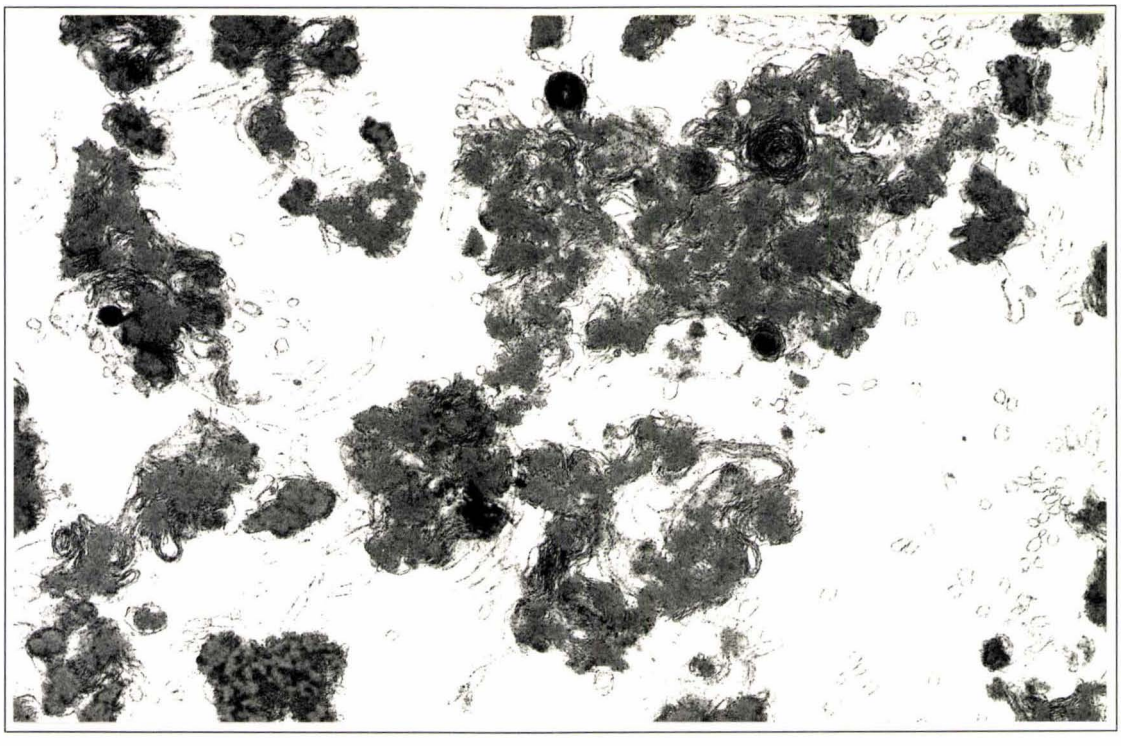


Figure 6-7: *Electron Micrograph of Subunit c Proteolipid* x 113,300.

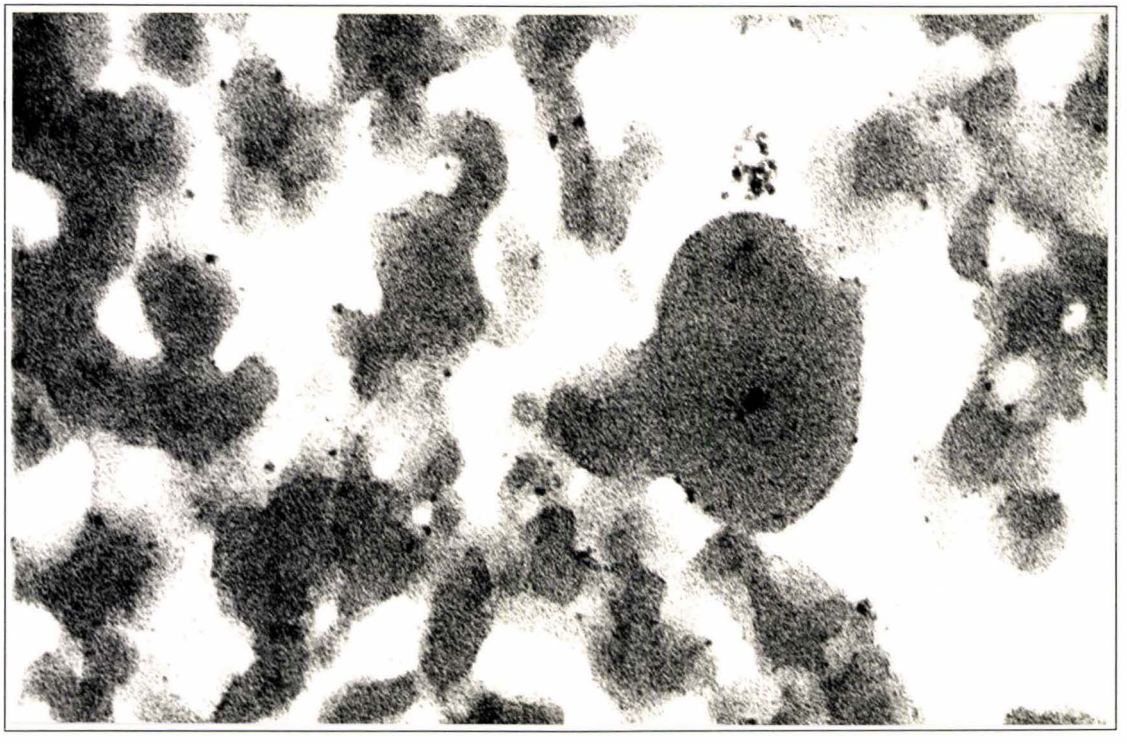
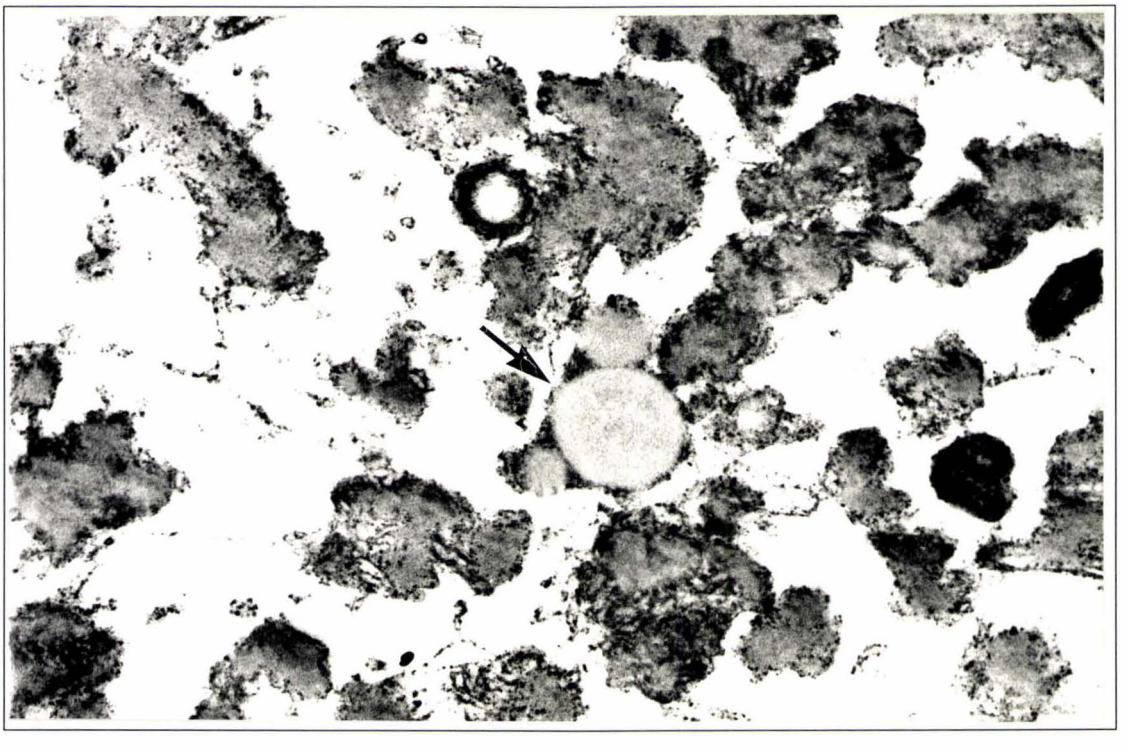


Figure 6-8: *Electron Micrograph of Liposomes made from Egg Phospholipids and Subunit c Proteolipid (1:1) Showing a "Lipid-droplet" like Structure (arrow)* x 33,400.



consistently displayed *in situ* and after extraction in various solutions. In general, for any single molecule, fluorescence can vary widely depending on the influences of any factor which changes the energy level of the absorbing orbital (e.g. ionisation with pH changes and hydrogen bonding) and any other factors that influence the rate at which the energy can be dispersed by vibration compared with the time required for the fluorescence decay. Thus the effects which influence the differences in energy between the ground and electronically excited states of molecules in mixed solvents are potentially very complex.

The major component of storage bodies in the ovine and late infantile human forms of ceroid-lipofuscinosis is the protein, subunit c of mitochondrial ATP synthase. Nearly all proteins are capable of fluorescence after irradiation with ultraviolet light due to aromatic amino acid residues, especially tryptophan (emission$_{max}$ 330-340nm) and tyrosine (emission$_{max}$ 303nm) but also to a lesser extent phenylalanine (emission$_{max}$ 258 and 282nm). The tertiary structure of proteins can also have a major effect on the observed fluorescence (Chen 1990). Proteins in solution are dynamic, constantly moving structures and thus the uniqueness of protein tertiary structures may be compromised in solution. The ultraviolet absorption spectra of total storage bodies and of the proteolipid in solution showed an absorbance maximum at 275, which is slightly less than the traditional 280 peak associated with protein absorption. This observation is consistent with the amino acid composition of subunit c and the lack of tryptophan in this molecule (Sebald and Hoppe 1981).

Chloroform/methanol or LDS solutions of ceroid-lipofuscinosis storage bodies, or their components, were not fluorescent. The ultraviolet-visible spectra of these solutions showed only a very small absorption in the 400 nm region, the common excitation region claimed to excite the characteristic fluorophor. Without absorption of energy there is none to be radiated at a longer wavelength which is the requirement for fluorescence, hence the storage bodies examined did not contain a single characteristic fluorophore of the type postulated. A concentration of 1.38 mg/ml redissolved liver storage body proteolipid gave a weak spectrum with an emission maximum of 500nm and a smaller peak at 680nm, using an excitation maximum of 400nm. These emission and excitation maxima peak heights were equalled by those of a 0.1 µg/ml solution of quinine sulphate (excitation maximum 350nm, emission maximum 450nm). This means that the storage body components in solution were 13,800 times less fluorescent than the quinine sulphate standard.

It is possible that microscopic autofluorescence may not require a strong intrinsic fluorophore. It is frequently observed that molecules exhibit higher fluorescence quantum yields when

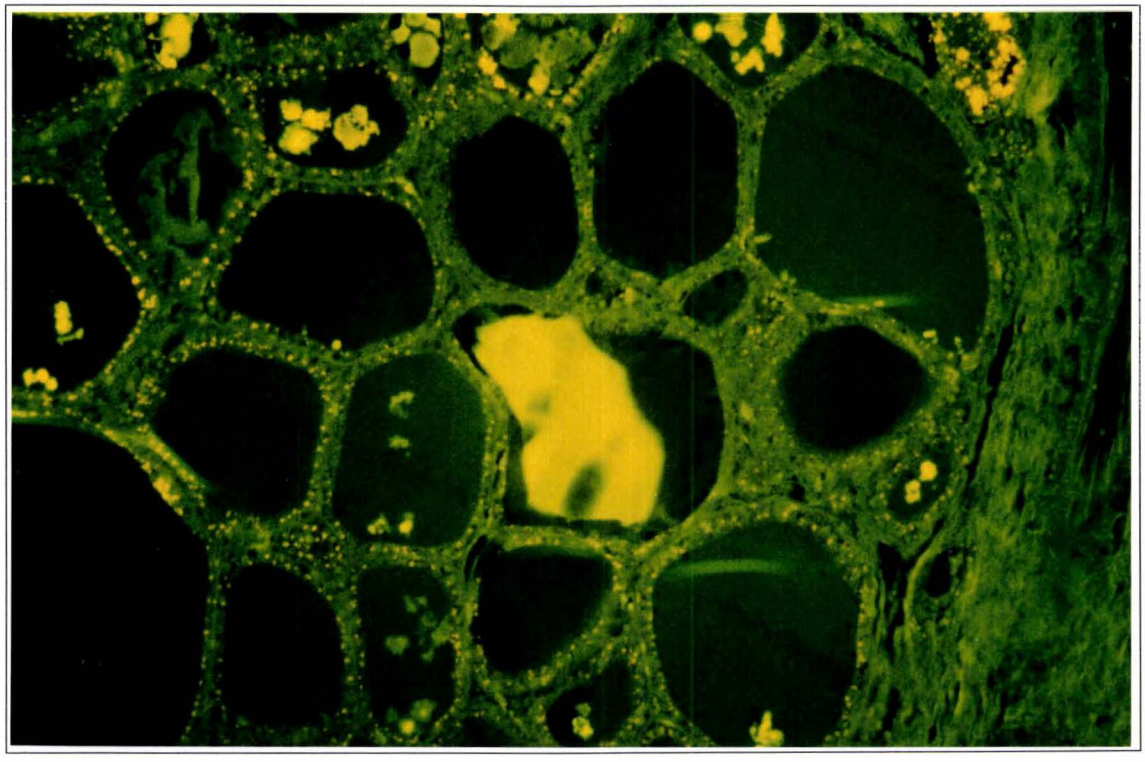
incorporated in solids, rather than in liquid solution (Wehry 1990). This effect is due to the protein tertiary structure and solvent interactions with solute molecules, following excitation in solution. The immediate environment of a particular solute molecule in a rigid solid is virtually constant, whereas it changes continually in liquid solution. Quenching is the name given to the effect where collision of the excited fluorophore with other molecules drains off the energy of the excited state. In some cases this results from very specific molecular interactions, for example quinine fluoresces in sulphuric acid but not in hydrochloric acid because collisions with chloride ions quench its fluorescence.

Protein conglomerates in histological tissue sections may show autofluorescence. This can be demonstrated in hepatic globules of the protein alpha-1-antitrypsin which show moderately intense blue-white autofluorescence under ultraviolet light and yellow-green autofluorescence under blue light when examined microscopically (DeLellis *et al* 1972). Additionally, colloid in the equine thyroid gland, which consists of the protein thyroglobulin, may undergo age-related degenerative changes showing as a basophilia of colloid. Under UV or blue light this colloid showed yellow, or yellow-green autofluorescence in unstained sections (Figure 6-9) (Dalefield *et al* 1994). This demonstrates that slight *in vivo* alterations in protein structure may lead to observable microscopic fluorescence within tissue sections.

Most fluorescence studies on the ceroid-lipofuscinoses have concentrated on detecting fluorophors in chloroform/methanol based extracts. Katz *et al* (1988) reported that the insoluble material remaining after chloroform/methanol extraction of canine storage granules was fluorescent but Palmer *et al* (1986a) reported that the residual material from ovine tissues was not fluorescent. Ovine storage bodies solubilised in LDS show virtually no absorption in the 400-500nm region and visually lose their fluorescence, compared with when they were suspended in water or water/glycerol. Analyses of storage bodies have shown that the dominant species (50-70% by weight) is protein and the identification of other major constituent groups does not leave room for any quantitatively significant unidentified fluorescent constituent.

Subunit c is thought to form a "hairpin" in the inner mitochondrial membrane, with hydrophobic amino acid residues within the membrane and hydrophilic residues on the membrane surface. A molecule containing both a nonpolar hydrophobic chain and one or more polar, hydrophilic functional groups is called an "amphiphilic" molecule. Within this context LDS, egg yolk and liver phospholipids and subunit c are all amphiphilic molecules. Such molecules may form aggregates of colloidal dimensions ("micelles") in liquid

Figure 6-9: *Fluorescence of Altered Colloid in a Thyroid Gland from an Aged Horse* x 100.
Fluorescence Microscopy, Excitation Filter 440nm with 530nm Barrier Filter.



solutions. Micellar systems are complex, dynamic systems, that are by nature heterogenous. There are inherent problems associated with the interpretation of fluorescence of molecules in such systems as the fluorescence properties of a molecule will depend on the specific microenvironment seen by that molecule during its excited state lifetime. The introduction of a fluorescent molecule into the interior of a micelle may bring about significant alterations in the fluorescence spectrum, quantum yield, and decay time (Wehry 1990). Subunit c is highly hydrophobic in nature and consequently has a high detergent to protein ratio in LDS micelles for polyacrylamide gel electrophoresis (PAGE), hence the apparent molecular weight of 3.5 kDa (Palmer *et al* 1988, 1992). The arrangement of subunit c in LDS micelles and in phospholipid micelles (liposomes), could be expected to be radically different, hence the fluorescence properties observed could be expected to be at variance. These differences could only be subjectively evaluated under a fluorescence microscope or under UV light, as during attempted quantitative measurement of phospholipid micelle turbid suspensions, artefacts occurred due to scattering of incident and fluorescence beams. Under the electron microscope the phospholipid liposomes with subunit c had a structural resemblance to storage bodies.

Other proteins have been studied as models of membrane proteins, or proteins that act on membranes. Melittin is a 26 residue peptide isolated from bee venom in which the N-terminal 6 amino acids are polar but the remainder of the molecule is hydrophobic. This protein can exist in a monomeric form or as a tetrameric aggregate in solution and contains one fluorescent tryptophan residue (Brown *et al* 1980, Lauterwein *et al* 1980). The fluorescence emission spectrum of melittin is very sensitive to interaction with some phospholipids (especially unsaturated lecithins), with the fluorescence spectrum changing to shorter wavelengths when this interaction occurs (Mollay and Kreil 1973). The self-association of melittin is thought to be accompanied by major conformational changes in the secondary structure of the peptide units involved (Talbot *et al* 1979). NMR spectral studies have suggested that melittin forms similar conformations when aggregated, and when micelle-bound (Brown *et al* 1980).

This study was undertaken because the observation of microscopic autofluorescence in the ceroid-lipofuscinoses has lead to much speculation that the chemical species responsible for the fluorescence must be central to the pathogenesis in this group of diseases. There is a vast discernible discrepancy between the sparkling fluorescence in paraffin tissue sections and the results of quantitative analyses of isolated components of ceroid-lipofuscinosis storage bodies in chloroform/methanol/ammonium acetate and LDS solutions which are not

fluorescent. Biochemical analyses by Palmer *et al* (1986a, b) preclude any quantitatively significant unidentified fluorescent constituent occupying more than 2-3% by weight of the storage body mass. The phenomenon of autofluorescence in the ceroid-lipofuscinoses is complex but it is concluded that there is no major fluorescent chemical species that is central to the pathogenesis of this disease other than protein.

Summary

A characteristic feature of the storage material in ceroid-lipofuscinosis is that it shows autofluorescence, either *in situ* or when isolated bodies are in suspension. However, when these storage cytosomes were dissolved the fluorescence was lost. The only significant absorbance spectra for dissolved storage bodies was at 275 nm which was in the range expected for protein. Emission and excitation maxima peak heights of the dissolved storage bodies were low, being 13,800 times less fluorescent than the quinine sulphate standard. It was concluded that the inherent fluorescence of storage material was associated with the tertiary arrangement of protein in its particular environment.

Chapter Seven

Raising Antibodies to Subunit c and Immunocytochemistry

Introduction

Monoclonal and polyclonal antibodies have proved to be useful tools in the study of the F_1F_0-ATP synthase complex of mitochondria, chloroplast's and bacterial cell membranes (Tzagoloff and Meagher 1971, Sebald and Hoppe 1981, Sanadi 1982, Amzel and Pedersen 1983, Vignais and Satre 1984, Hatefi 1985, Senior 1985, Godinot and Di Pietro 1986, Gautheron and Godinot 1988). An intramembrane component of the F_0 portion of this complex, subunit c, is the main constituent of the material stored in some forms of ceroid-lipofuscinosis (Palmer *et al* 1989, 1990, 1992, Fearnley *et al* 1990, Martinus *et al* 1991, Jolly *et al* 1992, Faust *et al* 1994). Despite years of intensive study of ceroid-lipofuscinosis (Batten disease) using available human tissue and animal models, the underlying biochemical anomaly in the disease remains unknown. It has been postulated (Palmer *et al* 1995) that subunit c storage diseases arise from different mutations affecting a specific degradation pathway of subunit c. The normal degradative pathway of subunit c is unknown. Antibody studies could be useful in the elucidation of this pathway. In addition, diagnosis of Batten disease is difficult requiring invasive tissue sampling and histological staining. No stain is highly specific for the storage of subunit c. Diagnosis of ceroid-lipofuscinosis has been attempted by quantitative measurement using an ELISA antibody method to detect subunit c extracted from urine (Wisniewski *et al* 1994) and various tissue homogenates but the specificity of the results of such tests is controversial.

Subunit c is a very hydrophobic transmembrane protein which is highly conserved between species. The amino acid sequences are identical in cattle, sheep and human beings (Medd *et al* 1993) and are similar to the equivalent protein in prokaryotic membranes (Sebald and Hoppe 1981). To elicit an antibody response prolonged immunisation schedules, consisting of up to nine immunisations, have previously been required (Loo and Bragg 1982) and working concentrations of polyclonal sera are reported to range from 1:100 to 1:500.

Antibodies have potential applications for improving the specific diagnosis and determining the pathogenesis of subunit c accumulation in Batten disease. Additionally, they may aid in the elucidation of normal mitochondrial proteolipid degradation. This chapter describes the production and characterisation of antibodies produced to subunit c by a variety of methods and describes the use of these antibodies for immunocytochemistry.

Special Materials and Methods

Tissue Fixation and Processing for Immunocytochemistry

Both perfusion and immersion techniques were used for ovine tissue fixation. For perfusion fixation, animals were deeply anaesthetised using 30 mg/kg sodium pentobarbitone. Carotid arteries and jugular veins were isolated, the head was severed from the carcass and a solution of 0.1% glutaraldehyde and 4.0% paraformaldehyde in 0.1M phosphate buffer was perfused into the carotid arteries. The brain and eyes were removed from the skull and post-fixed for 2-5 hours in perfusion solution and then stored at 4°C in 0.1M phosphate buffer with 0.01% calcium chloride. Tissues were also fixed by immersion in 10% neutral buffered formalin for varying times followed by immersion for the same time in 0.1M phosphate buffer at 4°C, before processing into paraffin wax.

Tissues were obtained by biopsy or at postmortem from cases of human late infantile Batten disease. These tissues were immersion fixed in 10% neutral buffered formalin for approximately 24 hours prior to embedding in paraffin wax. Archival blocks of paraffin-embedded tissue were obtained from adult onset (Kuf) patients, English setter, Chihuahua, Border collie, Tibetan terrier, miniature Schnauzer, Corgi and Cocker spaniel dogs and from Devon cattle and Swedish lambs. Fixation times for these tissues were unknown.

Note: Paraffin blocked brain sections from three patients with Kuf disease were supplied by Dr. L.S. Wolfe and Dr. J.H. Deck, Mc Gill University, Montreal, Canada; Border collie tissues by Dr. V.P. Studdert, University of Melbourne, Australia; English setter tissues by Dr. F. Lingaas, Norwegian College of Veterinary Medicine, Norway; Golden retriever tissues from Dr. J.S. Patterson, University of Michigan, USA and brain from the miniature Schnauzer from Dr. R.H. Sutton, University of Queensland, Australia.

Antigen Preparation

Subunit c proteolipid was extracted from isolated liver storage bodies by chloroform:methanol:ammonium acetate extraction (see general materials and methods, chapter three). The extracted proteolipid was precipitated with diethyl ether at -70°C, then washed with methanol and distilled water. The concentration and purity of the sample was determined by quantitative amino acid analysis. Some of the washed proteolipid was cleaved with cyanogen bromide (CNBr). This cleavage was carried out by dissolving the washed proteolipid in distilled 100% formic acid containing 100 mg/ml CNBr (Martinus 1990). The resulting solution was gassed with nitrogen and left in the dark at 25°C for 24 hours. Solvents were removed by drying *in vacuo* over NaOH pellets and the digest was stored at -20°C until required.

Proteolipid or CNBr Digest in Freund's Adjuvant

The washed ether precipitate of proteolipid or the products of CNBr clevage were dissolved in a small volume of trifluroacetic acid (approximately 5 µl trifluroacetic acid for 1 mg protein) then dried down over NaOH pellets *in vacuo.* The precipitates were resuspended in PBS to give approximately 2 mg/ml protein, sonicated and mixed with an equal volume of Freund's adjuvant (complete or incomplete) to make a stable suspension of 1 mg protein/ ml final volume.

Proteolipid or CNBr Digest in a Liposomal Adjuvant

Washed ether precipitated proteolipid (4 mg) or the products of CNBr proteolipid digestion, were dissolved in 20 µl of trifluroacetic acid then transferred into a vial that contained RIBI adjuvant (0.5 mg of trehalose dimycolate, 0.5 mg monophosphoryl lipid A, 0.5 mg of mycobacterium cell wall skeleton in oil and Tween-80; RIBI Immunochemical Research Inc., Hamilton, Montana). The trifluoroacetic acid was dried off *in vacuo* over NaOH pellets. When the contents were dry, the vial was warmed to 40°C for 10 minutes and 2 ml sterile 145 mM NaCl was added. The vial contents were mixed in a vortex mixer then sonicated for 30 seconds, prior to storage at 4°C until required.

Inoculation of Animals and Collection of Antisera

New Zealand white rabbits and broiler chickens (3 months of age) were inoculated subcutaneously with 2 mg of antigen in 1 ml of Freund's complete adjuvant or with 1 mg of antigen in 0.5 ml of RIBI adjuvant in one or two different sites on the back. Booster injections of 1 mg of antigen in 0.5 ml of Freund's incomplete adjuvant or of 0.5 mg of antigen in 0.25 ml of the RIBI adjuvant were given 21-28 day intervals until an immune response was detected. Blood samples were taken from the brachial wing vein (chickens) and the marginal ear vein, or the central ear artery (rabbits) prior to the first inoculation and 10 days after each subsequent immunisation. After an immune response was detected, blood samples were taken at varying intervals but at least a week apart. Further booster injections were given when the immune response fell.

Other Antisera

Additional Antisera obtained are listed in Table 7-1.

Table 7-1: *Source of Antisera and Description of the Antigen used in their Preparation.*

Source of Antibody	**Antigen used in the Preparation of Antibody**
Dr. E. Kominami	Synthetic NH_2 terminus of subunit c residues 1-11.
Dr. D.N. Palmer	Synthetic NH_2 terminus of subunit c residues 1-11, Hydrophilic loop region of subunit c residues 34-44.
Chemicon*	65 kDa human mitochondrial protein.
Dr. Y. Hatefi	Beef heart DCCD binding proteolipid.
Dr. R. Allardyce	Sheep liver storage body proteolipid.
Dr. A. Hille-Rehfeld	Human β-hexosaminidase.

*Chemicon International Inc., Temecula, California, USA.

ELISA Test

The antibody titres of the antisera raised were determined by an ELISA test. This was carried out by diluting a chloroform-methanol solution of subunit c to contain at least 70% methanol to avoid damage to the ELISA plate. A 100 µl aliquot of this solution containing 1 µg of subunit c was placed in each well of a Falcon 3912 microtest III flexible assay plate (Becton Dickinson Labware, Oxnard, California, USA) and the solvent was dried off. Several different brands of ELISA plates were tried as some brands of microtitre plates reacted with high concentrations of antibody in the absence of subunit c coating. The coated plates were blocked with 3% coffee whitener (DYC Foods, Auckland, NZ) in PBS for 1 hour, washed three times with PBS and incubated with 100 µl per well of serum diluted with PBS containing 0.1% Tween 20 (PBST) and 3% coffee whitener for 1 hour. Serum dilutions screened were 1/10, 1/50 with sequential two fold dilutions thereafter. Plates were then washed three times with PBST prior to 100 µl biotinylated antispecies antibody diluted 1/ 10,000 with PBST being added to each well and incubated for 1 hour. Plates were washed a further three times with PBST and incubated for 1 hour with 100 µl per well of streptavidin biotinylated horse radish peroxidase complex (Amersham International Inc., Buckinghamshire, UK) diluted 1/16,000 with PBST. After another three washes with PBST, the colour was developed with a solution of 4 mM o-phenylenediamine and 0.04% v/v H_2O_2 in 0.1 M citric acid 0.2 M phosphate buffer, pH 5, for 15 minutes in the dark. The reaction was stopped by the addition of 100 µl of 1 M H_2SO_4 and the absorbance read at 492 nm in a 340 ATC ELISA reader (SLT Lab Instruments, Salzburg, Austria).

PAGE and Western Blotting

Proteins were separated on LDS/polyacrylamide gels containing 15% polyacrylamide that were 1.5 mm thick (Laemmli 1970) then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA). Protein transfer to the PVDF membrane was carried out in a mini Trans-Blot cell (Bio-rad Laboratories, Hercules, CA, USA) for 45 minutes with a current of 250mA and 15V in a solution of 25 mM Tris and 192 mM glycine in 20% methanol (see general materials and methods, chapter three). Proteins adsorbed to the membrane were detected using either colloidal gold total protein stain (Bio-Rad Laboratories) or Coomassie Blue in aqueous solution (Gültekin and Heermann 1988). After blotting the membrane was rinsed in distilled water, dried between sheets of blotting paper and stored at -20°C until required.

Immunostaining of the PVDF Membrane

Staining of the PVDF membrane was carried out either by enhanced chemiluminescence as described in chapter three or by the following staining procedure. All steps were done at room temperature with gentle agitation. The unoccupied protein binding sites in the membrane were blocked by incubation in 3% BSA in PBST for 1 hour. This was followed by three 10 minute washes in PBST. At this stage, depending on the number of antisera being used, the membrane was either incubated in one reagent solution (approximately 10 ml) or it was placed in a 15 slit plate as described by Gültekin and Heermann (1988). Approximately 500 µl reagent solution was used per slit. Incubation in the primary antisera was carried out for 1 hour, then the membrane was rinsed three times for 10 minutes each with PBST. This was followed by an incubation in biotinylated antispecies IgG, diluted 1:500 for rabbit sera or 1:1000 for chicken sera for 1 hour and another three rinses for 10 minutes each in PBST. The membrane was then incubated in streptavidin biotinylated horse radish peroxidase diluted 1:1000 for 1 hour and rinsed three times in 10 ml PBST as previously. Detection of antibody binding was by enzymatic staining with 10 ml of 0.05% 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB) (Sigma Chemical Co) in PBS with 10 µl 30% hydrogen peroxide (BDH limited) and 10 µl of a 1% nickel and cobalt chloride solution, for 10 minutes. Colour development was terminated by washing the membrane with distilled water.

Immunocytochemistry of Paraffin Embedded Tissue Sections

All immunocytochemistry incubations were carried out at room temperature in a humidity chamber. Sections from paraffin embedded tissues were cut at 5 µm thickness, placed on double washed microscope slides, dewaxed and brought to water. This was followed by two washes for 5 minutes each in phosphate buffered saline (PBS) and a preincubation for 1 hour in 1% normal goat serum (NGS) in PBS to block non-specific protein binding sites. The sections were then incubated in anti-subunit c rabbit sera (RabA) diluted 1:2,000, anti-NH_2-terminal peptide of subunit c (from Dr. E. Kominami) diluted 1:500, anti-NH_2-terminal peptide of subunit c (from Dr. D.N. Palmer) diluted 1:100, anti-β-hexosaminidase diluted 1:200 or anti-hydrophilic loop region of subunit c diluted 1:20. The diluent was 1% NGS in PBS and the sections were incubated in the primary antibody solution for 2 hours. After three washes for 1 minute each in PBS the sections were incubated in biotinylated anti-rabbit IgG diluted 1:200 in 1% NGS in PBS for 30 minutes. Sections were again washed

three times for 1 minute each in PBS, then incubated for 30 minutes in avidin biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, California, USA) made according to manufacturers instructions. This was followed by a further 3 washes for 1 minute each in PBS. Colour development occurred by reaction in a 1 mg/ml solution of 3,3'-diaminobenzidine tetrachloride containing 0.02% H_2O_2 v/v in PBS for 6-10 minutes. Sections were counterstained with Mayers haematoxylin before dehydration and mounting using DPX mountant. Control sections were treated by the same procedure except that the primary antibody was omitted, or replaced with pre-immune rabbit sera.

Immunocytochemistry of "Vibroslice" Sections

Blocks of perfusion-fixed tissue were cut at 35 μm on a "Vibroslice model 752" (Campden Instruments Ltd., U.K.). Sections were pre-treated with 0.6% hydrogen peroxide in methanol to inactivate endogenous peroxidases and incubated for 1 hour in 1% NGS in PBS. For some sections 0.05% triton X-100 (BDH Chemicals Ltd., Poole, England) was included in this NGS incubation, in subsequent washes and in the primary antibody and bridge solutions. Primary antibodies were used at 1:10,000 for RabA antibody, 1:500 for anti-mitochondrial antibody (Chemicon), or 1:2,000 for anti-NH_2-terminal peptide (from Dr. E. Kominami) during overnight incubation at 4°C. Incubation times for the bridging antibody (biotinylated anti-rabbit IgG, as recorded above, or biotinylated anti-mouse IgG), and avidin biotinylated horseradish peroxidase complex (as above) were 30 minutes, and were preceded by three washes in 0.1% NGS in PBS. A final three washes were carried out in PBS alone, then peroxidase label was demonstrated using 0.05% 3,3'-diaminobenzidine tetrachloride with 0.01% hydrogen peroxide in PBS for 10 minutes in the dark. Sections for light microscopic study were dehydrated and mounted on 1% gelatin-coated microscope slides, with and without H & E staining. For electron microscopic study a section was cut into squares less than 1 mm in size and flat embedded on glass slides in araldite-epoxy resin. After polymerization these sections were remounted on epoxy resin blanks and cut into thin sections.

Cytochrome Oxidase Histochemistry

Vibroslice sections (as described above) were incubated for 1-2 hours in 0.05 M PBS pH 7.4 containing 0.5 mg/ml 3,3'-diaminobenzidine tetrachloride, 2 μg/ml catalase, 1 mg/ml

Cytochrome C (Sigma, type II), 75 mg/ml sucrose and 4 µl/ml of a 1% cobalt chloride and 1% nickel chloride solution. The sections were then washed in distilled water, dehydrated and mounted on 1% gelatin-coated microscope slides, with and without H & E staining. Control sections were incubated in medium, as above, with the addition of 0.65 mg/ml potassium cyanide.

Culture of Fibroblasts

Fibroblasts from normal sheep and sheep affected with ceroid-lipofuscinosis were obtained from a tissue bank. These were thawed, then re-cultured. During the growth phases, fibroblasts were cultured in Eagle's minimum essential medium pH 7.4 (Sigma Chemical Co., St. Louis, USA), a growth media containing 10% foetal calf serum and 1% PSK antibiotics (penicillin 100 units/ml, streptomycin 100 µg/ml and kanamycin 100 units/ml). Fibroblasts from ceroid-lipofuscinosis affected sheep were re-cultured for six passages, while fibroblasts from normal sheep were cultured for eight passages. To passage cells, the growth media was first discarded, the cells were rinsed twice in PBS at 37°C, then the cells were incubated in a solution containing 0.05% trypsin, 0.02% versene and antibiotics (as above) (ATV solution) at 37°C for 2 minutes, or until the fibroblasts were suspended in solution. Growth medium was added to counteract the effect of the trypsin, then aliquots of suspended cells were pipetted into new flasks containing growth medium. After the last growth phase during the final passage, the cells were maintained at confluence in minimum essential medium containing 2% foetal calf serum and 1% PSK antibiotics for 4 days.

Two different protocols were used to remove fibroblasts from the growth flasks. In the first protocol, fixative solution at 37°C was added to the culture flasks, then the cells were manually scraped off the culture surface. These cells in fixative solution were transferred by pipette to 1.5 ml tubes and pelletted by centrifugation. The cells were processed as described below. In the second protocol, the cultured fibroblasts were incubated in ATV solution at 37°C to remove them from the culture surface, then the resulting solution was transferred by pipette and centrifuged as above. The supernatant was discarded, fixative solution was added, and the cells were processed as described below.

Fixation and Embedding for Ultrastructural Immunocytochemistry

Tissue blocks (approximately 1 mm^3), or cells or material precipitated in small centrifuge tubes, were fixed in 1% paraformaldehyde containing 0.5% glutataldehyde in 0.1 M PBS, pH 7.2 for 2 hours at room temperature. Fixation was followed by a 30 minute incubation at room temperature in 0.05 M ammonium chloride in PBS (to block aldehyde groups), then a wash in 1% BSA in PBS for 15 - 18 hours. Tissue blocks were dehydrated in two changes of 50% ethanol for 15 minutes each, then four changes of 70% ethanol for 30 minutes each, before transferring into LR white resin (London Resin Co., Basingstoke, Hampshire). The resin mixes consisted of two changes of 1:1 100% ethanol to resin for 30 minutes, followed by three incubations in 1:2 100% ethanol to resin for 1 hour each, then four changes of pure resin followed by embedding in resin in gelatin capsules at 55°C for 24 hours. Ultrathin sections (80-100nm) were cut and mounted on 300 mesh nickel grids.

Immunogold Labelling

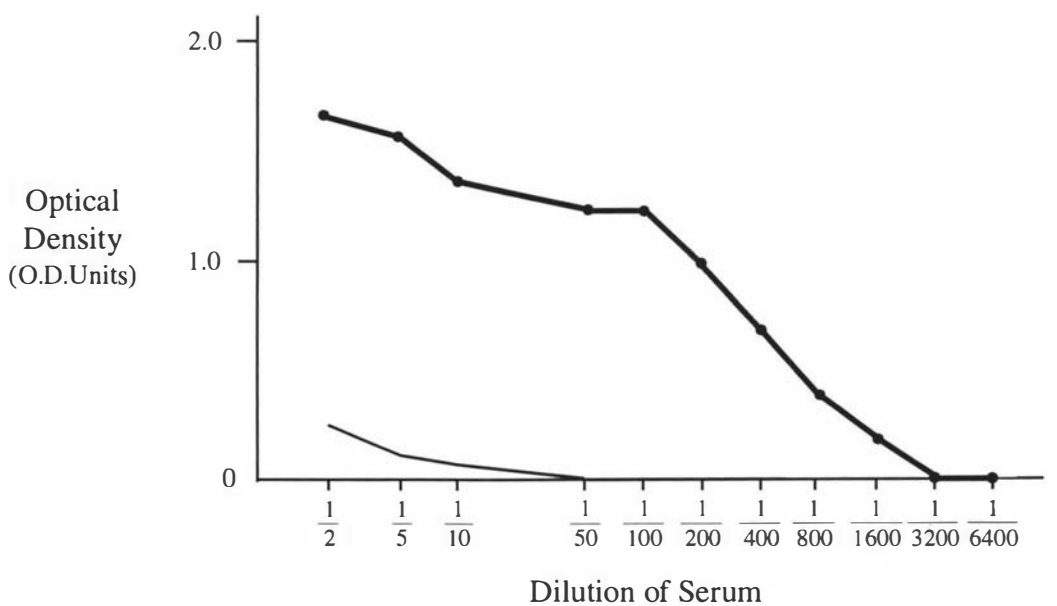
Incubations were performed by floating grids on droplets of reagent solutions on Parafilm. All incubations were carried out at room temperature unless otherwise stated. Grids were first transferred to distilled water for 10 minutes, followed by incubation in 5% normal goat serum (NGS) in Tris-buffered saline pH 8.0 (TBS) for 15 minutes. This was followed by incubation in primary antibody diluted in 1% NGS in TBS for 24 hours, at 4°C. The primary antibodies were diluted to 1:2,000 for RabA antibody, 1:2,000 for anti-NH_2-terminal peptide (from Dr. E. Kominami), 1:100 for anti-NH_2-terminal peptide (from Dr. D.N. Palmer) and 1:20 for anti-hydrophilic loop region of subunit c. After this incubation, grids were rinsed by 2 x 5 minute washes in 0.1% NGS in TBS, then labelled with 15 nm goat anti-rabbit IgG-gold (Auroprobe EM GAR IgG G15 Amersham International, Buckinghamshire England) diluted 1:25 in 0.1% NGS in TBS for 30 minutes. Sections were then washed for 2 x 5 minutes in 0.1% NGS in TBS, in distilled water for 5 minutes, then left to air dry. After immunolabelling, sections were first stained with saturated uranyl acetate for 10 minutes then rinsed with distilled water. Secondly, sections were stained for 10 seconds with saturated lead citrate which had been diluted 50% with distilled water. The sections were rinsed again with distilled water and left to air dry.

Results

Determination of Antibody Response

Antibody responses were monitored by an enzyme linked immunosorbant assay (ELISA) and immunostaining of Western blots. The highest antisera titre occurred in a rabbit (RabA) using proteolipid extracted from storage cytosomes in the RIBI adjuvant system. With this antigen in RIBI adjuvant, a recognisable immune response occurred in two innoculated rabbits 10 days after four injections at monthly intervals. This response increased after an additional three injections over 6 months and was at its maximum a month after each injection. One of these rabbits (RabA) however had a much higher immune titre than the other rabbit (RabB). An ELISA result is shown in Figure 7-1. No immune response was demonstrated in rabbits using the cyanogen bromide digest fragments in the RIBI system, or with the complete molecule or its cleavage fragments in Freund's adjuvant.

Figure 7-1: *An ELISA Assay Showing the Response in O.D. Units Measured at 492nm of a Rabbit Inoculated with Subunit c in RIBI Adjuvant. Successive Dilutions of Serum were Incubated with 1μg of Subunit c.*



Inoculation with complete subunit c proteolipid and the cyanogen bromide digest fragments in Freund's adjuvant resulted in antisera production after 6-7 immunisations in chickens. However, this antisera never reached the activity of the rabbit antisera from either RabA or RabB.

Immunostaining of Western blots with the antisera confirmed the ELISA results (Figure 7-2). Rabbits innoculated with the proteolipid in the RIBI system produced the most active antisera, which is consistent with the ELISA results. Monomeric subunit c and a 24,000 MW putative oligomer of subunit c were specifically stained in affected sheep liver and pancreas homogenates at the antisera dilution of 1:3,000. Storage bodies isolated from these tissues also contained a 14,800 MW putative oligomer. In control liver and pancreas homogenates, these bands were barely visible, but monomeric subunit c stained specifically in blots of normal mitochondrial proteins. Proteolipid extracts of storage bodies and normal mitochondria stained in the same way.

Immunocytochemical Staining Characteristics

Immunocytochemical staining of histological sections initially showed some positive results with RabA antiserum but the staining was very variable. Fixation regimes were therefore investigated. Different fixation protocols for ovine tissues prior to immunostaining with various anti-subunit c antibodies showed that immunostaining was highly sensitive to the time in 10% neutral buffered formalin. This could be reversed to a certain degree by washing in phosphate buffer pH 7.2. Optimum fixation was achieved by immersion in 10% neutral buffered formalin for 4 - 24 hours followed by washing in phosphate buffer, pH 7.2, for an equivalent period before embedding in paraffin wax. Perfusion fixation followed by vibroslice sectioning or paraffin embedding gave more intense immunostaining results than immersion fixation techniques.

Strong immunocytochemical staining of storage material was obtained in many different tissues (Figures 7-3, 7-4, 7-5), including cerebral cortex (Table 7-2), liver, kidney, pancreas and heart muscle in late infantile human and ovine ceroid-lipofuscinosis with antibodies raised against purified subunit c (RabA) and synthetic NH_2 terminal peptide of subunit c from Dr. E. Kominami. Variable but much lighter staining occurred in Devon cattle with ceroid-lipofuscinosis and various canine forms of the disease.

Figure 7-2: *Western Blot Developed using Serum from a Rabbit Inoculated with Subunit c in RIBI Adjuvant.*

The Lanes are:

(a) Normal Sheep Liver Homogenate;
(b) Affected Sheep Liver Homogenate;
(c) Normal Sheep Pancreas Homogenate;
(d) Affected Sheep Pancreas Homogenate;
(e) Liver Storage Bodies;
(f) Pancreas Storage Bodies and
(g) Normal Sheep Mitochondria.

(Courtesy of Miss S.L. Bayliss)

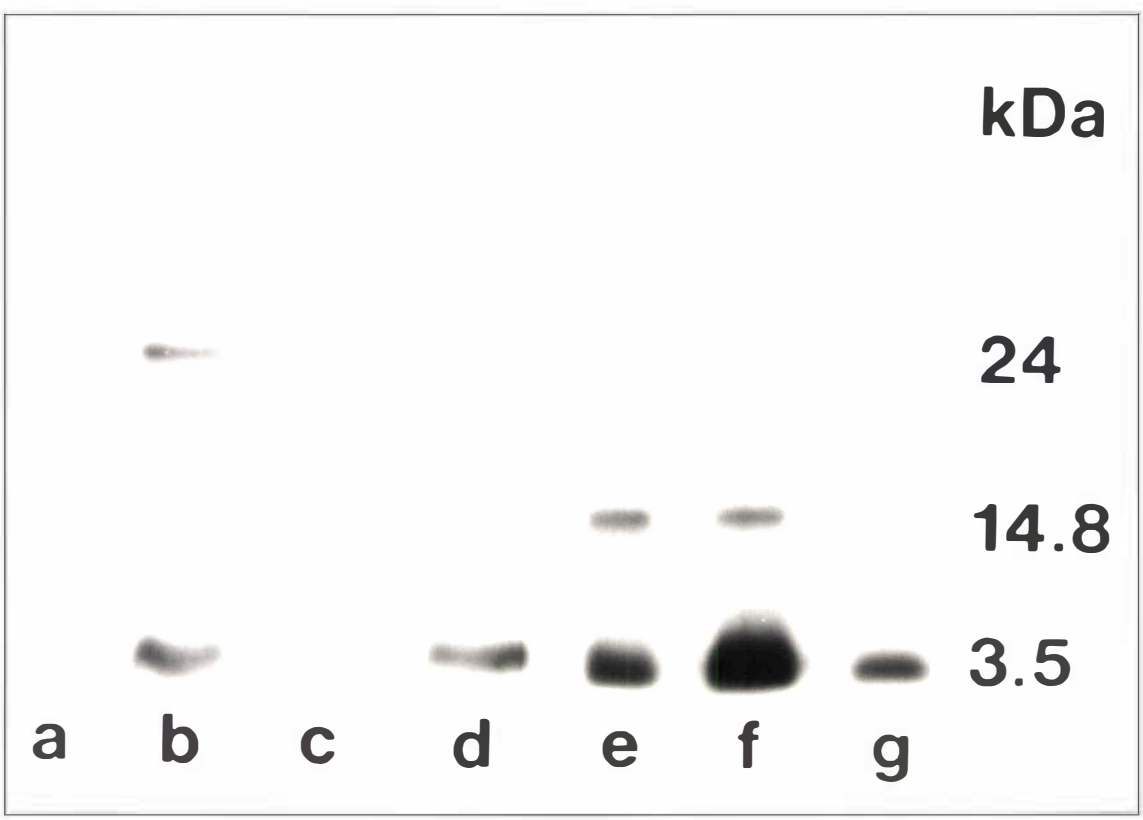
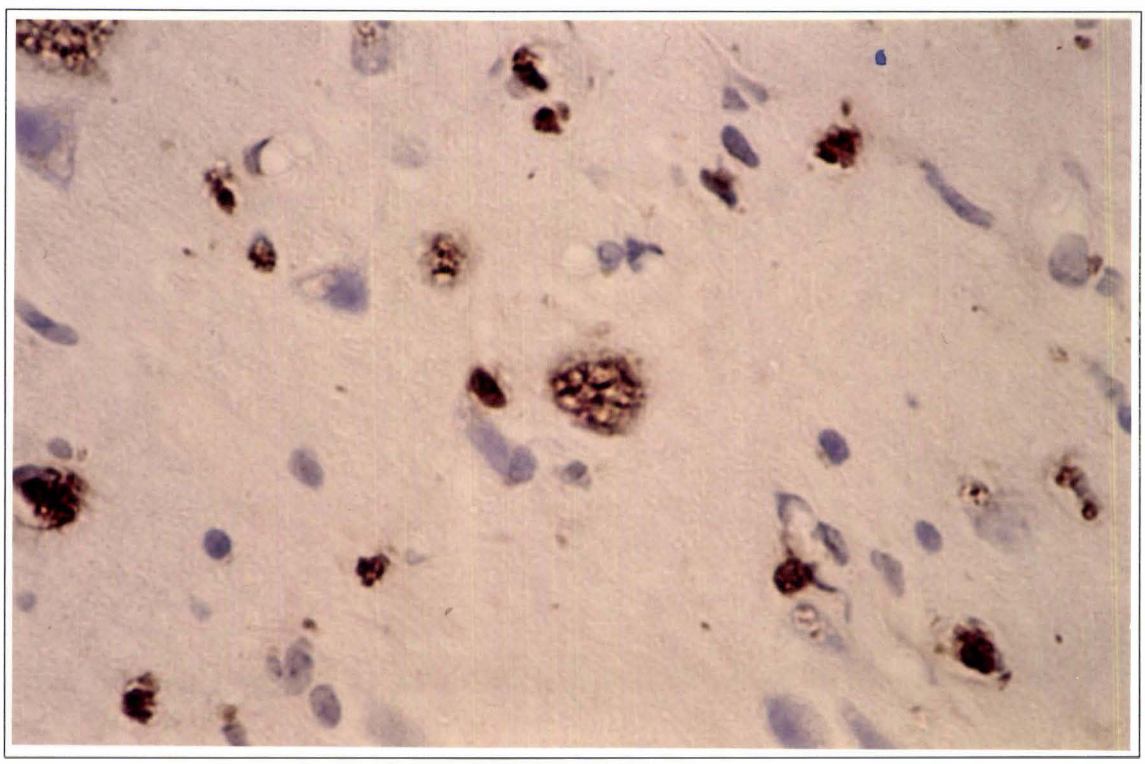


Figure 7-3: *Cerebral Cortex from*
(a) A Sheep Affected with Ceroid-lipofuscinosis and
(b) A Phenotypically Normal Sheep Immunostained using Subunit c Antiserum (RabA); Paraffin Sections x 250. *Haematoxylin Counterstain.*



(a) as shown above - (b) as shown below.

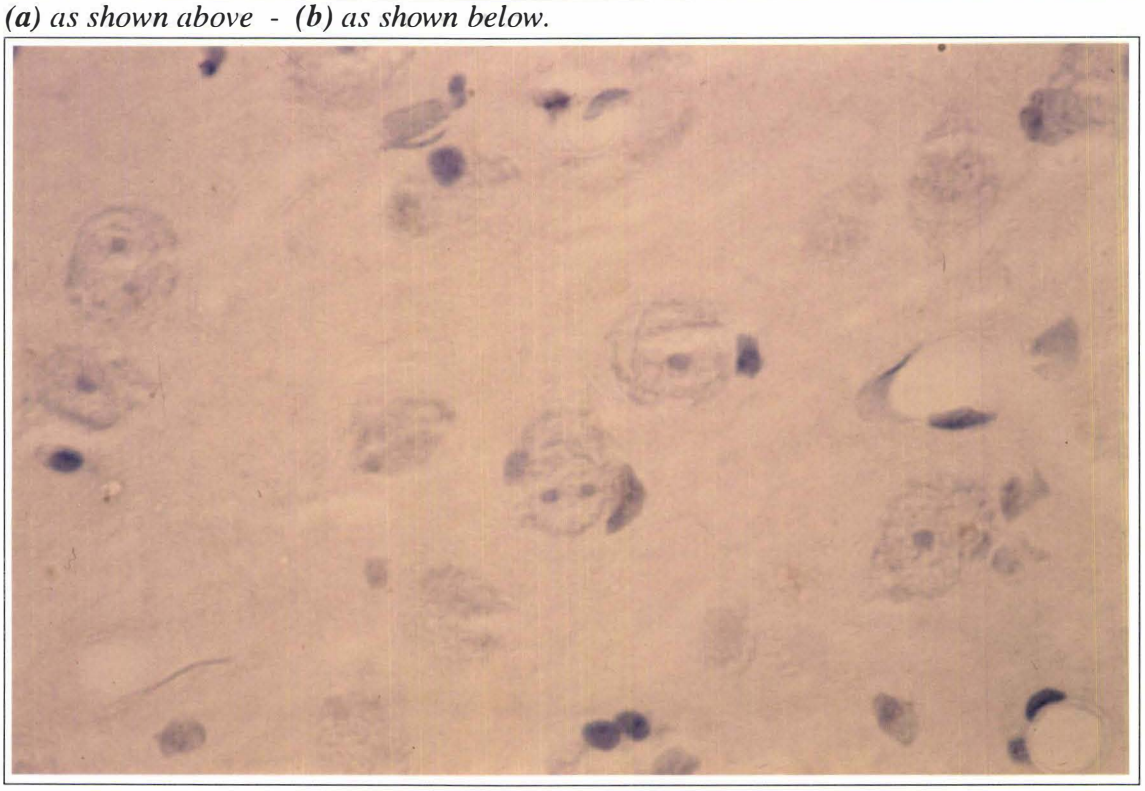
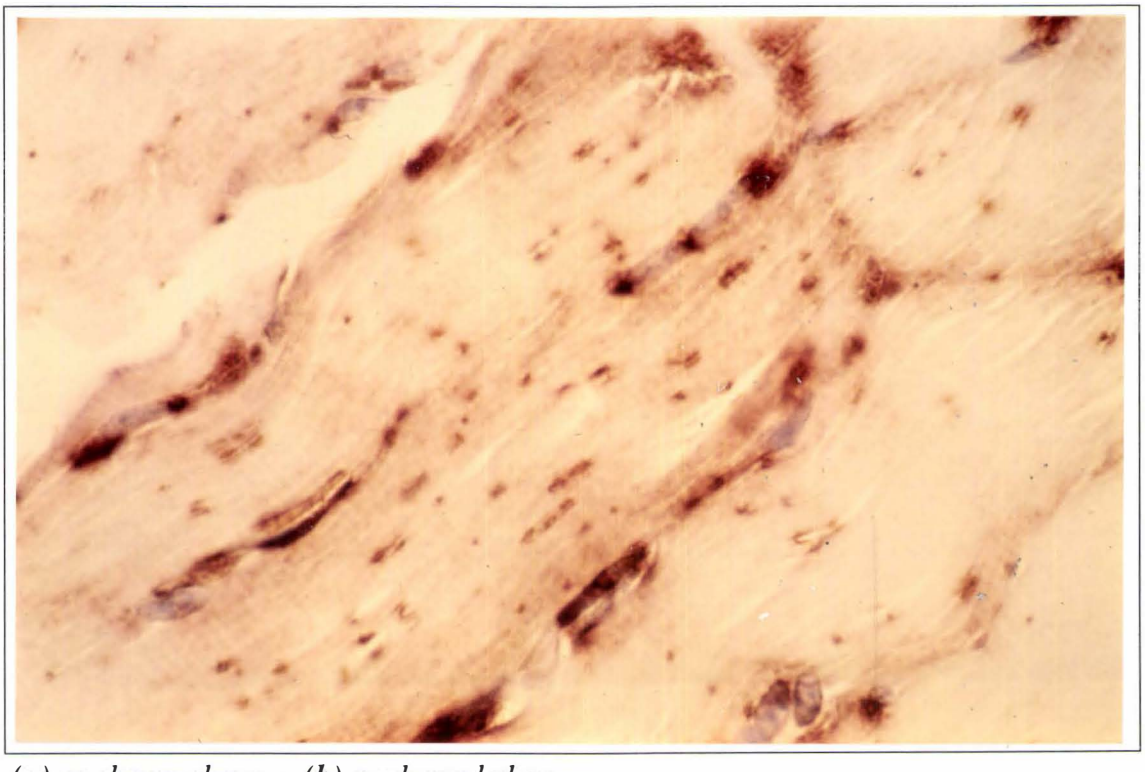
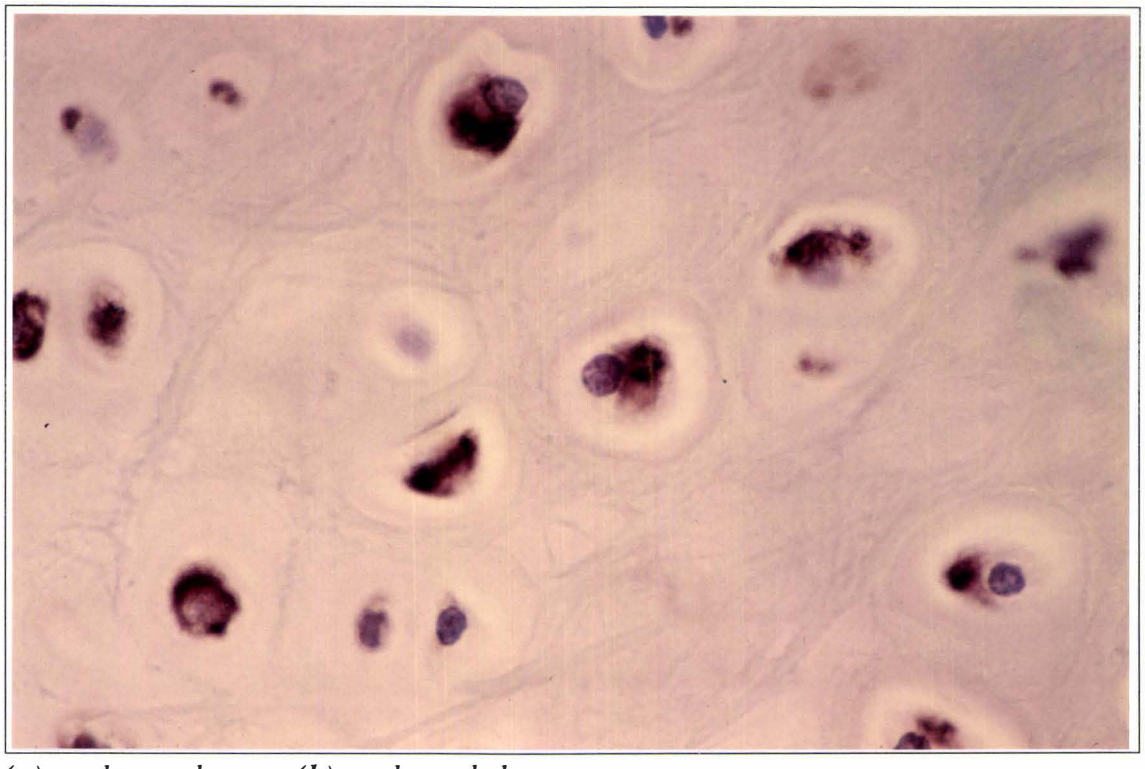


Figure 7-4: *Skeletal Muscle from*
(a) A Sheep Affected with Ceroid-lipofuscinosis and
(b) A Phenotypically Normal Sheep Immunostained using Subunit c Antiserum (RabA); Paraffin Sections x 250. *Haematoxylin Counterstain.*



(a) as shown above - (b) as shown below.

Figure 7-5: *Ear Cartilage from*
(a) A Sheep Affected with Ceroid-lipofuscinosis and
(b) A Phenotypically Normal Sheep Immunostained using Subunit c Antiserum (RabA);
Paraffin Sections x 250. *Haematoxylin Counterstain.*



(a) as shown above - (b) as shown below.

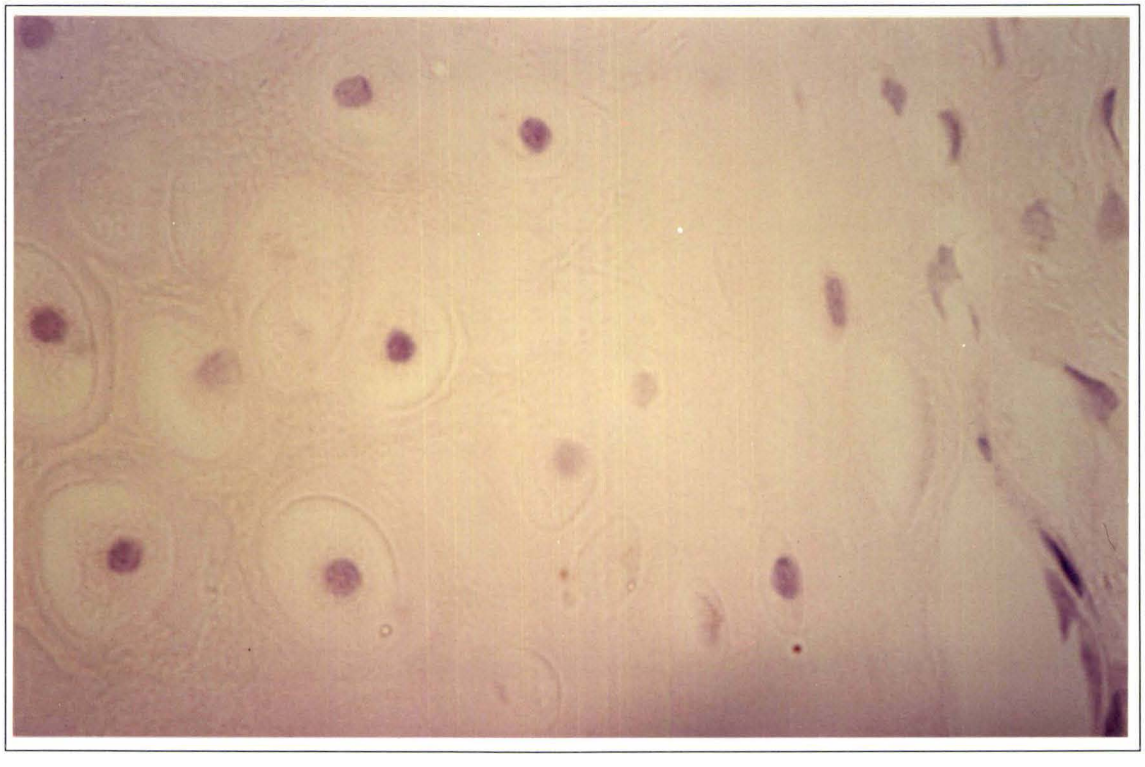


Table 7-2. *Immunocytochemical Staining of Cerebral Cortex using Antibodies to Semi-purified Subunit c and a Synthetic NH_2 Terminal Peptide of Subunit c.*

Form of Ceroid-lipofuscinosis	**Subunit c antibody**	**NH^2 terminal antibody**
Late infantile human	+++++	+++
Kuf	+	+++
Ovine	++++	+++
English setter	+	+
Border collie	+	+
Tibetan terrier	++ *(variable)*	O
Swedish lambs	+	+
Schnauser	O	O
Corgi	+ *(variable)*	O
Cocker spaniel	++	+
Golden retriever	+	O
Devon cattle	+	+

Storage bodies within cells from sheep tissues generally showed intense staining with RabA and synthetic NH_2 terminal peptide of subunit c from Dr. E. Kominami antisera but the staining was limited to the periphery of the cytosomes in neurons (Figure 7-3). Storage material within cells in human late infantile Batten disease was consistently intensely immunostained regardless of the cell type (Figure 7-6). In the ovine and late infantile human forms of Batten disease, immunocytochemical staining was more intense with the antibodies against purified subunit c, whereas in Kuf disease (Adult onset Batten disease) the staining was more intense with the antibodies raised to the synthetic NH_2 terminal peptide of subunit c (Figure 7-7). Staining of storage material in the cerebral cortex and other tissues (where available) was mild and variable in English setter, Border collie, Tibetan terrier, Corgi, Cocker spaniel and Golden retriever dogs affected with ceroid-lipofuscinosis. No staining was evident in tissue from the miniature Schnauzer dog.

The synthetic NH_2 terminal peptide antisera raised by Dr. D.N. Palmer stained storage bodies within sections from late infantile human and Kuf disease mildly to moderately well but ovine and canine storage bodies were only faintly stained. Sections stained with this antisera exhibited high non-specific background staining.

Sections stained using antisera raised to the hydrophilic loop region of subunit c again showed high background staining. Human late infantile storage bodies were stained to a mild to moderate degree, but ovine storage bodies remained unstained. Antisera raised to DCCD-binding proteolipid immunostained late infantile human, Kuf and ovine storage bodies to a mild to moderate degree but again showed high, apparently non-specific background staining.

Antisera to human β-hexosaminidase gave effective staining on the late infantile human storage bodies. No reaction was observed with ovine and canine sections.

Immunostaining of vibroslice sections of cerebral cortex from ceroid-lipofuscinosis affected sheep using an anti-mitochondrial antibody (Table 7-1) showed staining of some mitochondria which appeared enlarged (Figure 7-8). These enlarged mitochondria were also observed when cytochrome oxidase histochemistry was carried out.

Figure 7-6: *Cerebral Cortex from a Human Patient with Late Infantile Batten Disease Immunostained using Subunit c Antiserum (RabA); Paraffin Section* x 250. *Haematoxylin Counterstain.*

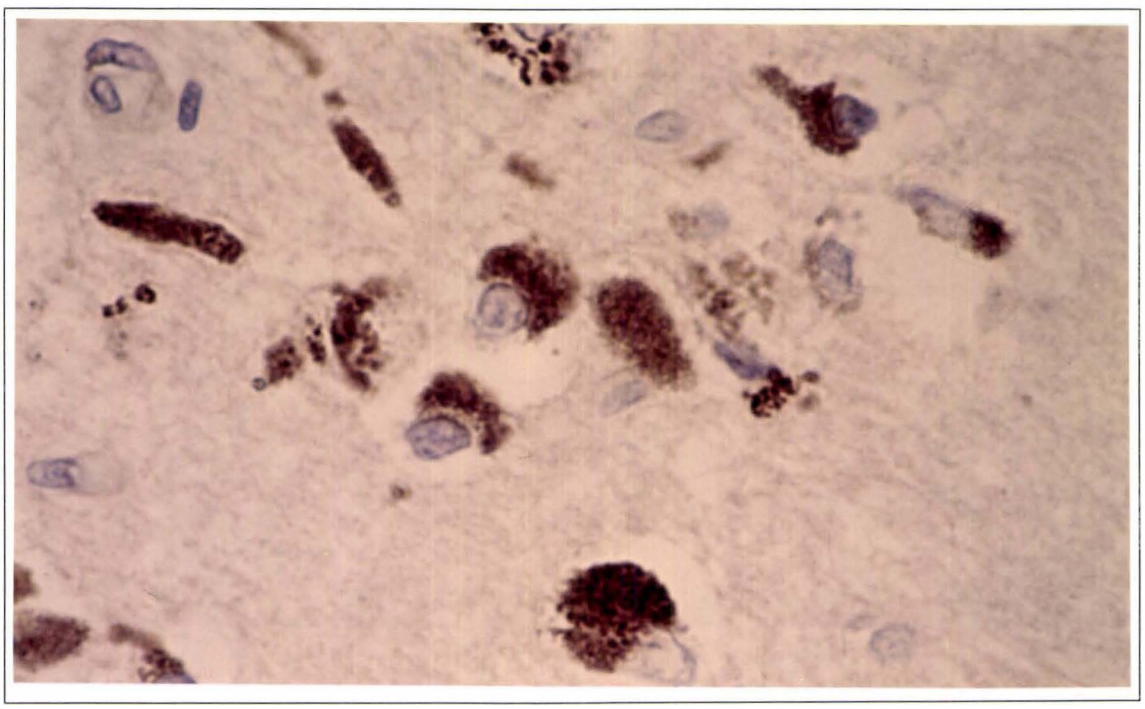


Figure 7-7: *Cerebral Cortex from a Human Patient with Kuf Disease (Adult Batten Disease) Immunostained using Antiserum to the Synthetic* NH_2 *Terminal Peptide of Subunit c from Dr. E. Kominami; Paraffin Section* x 250. *Haematoxylin Counterstain.*

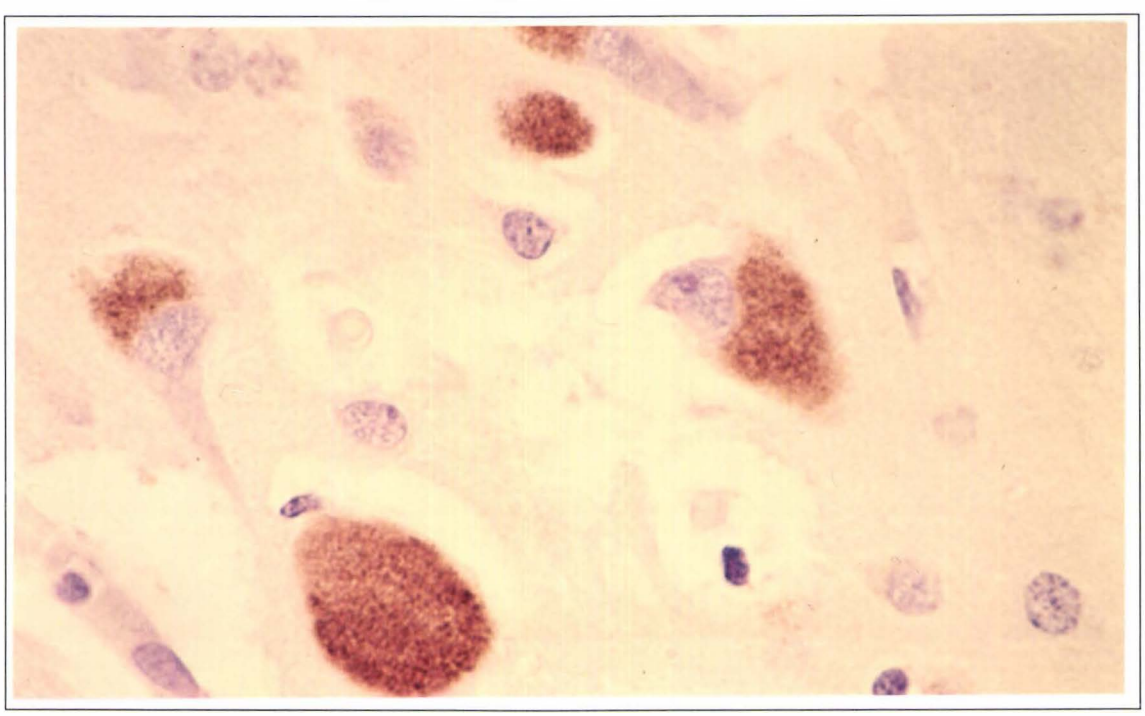
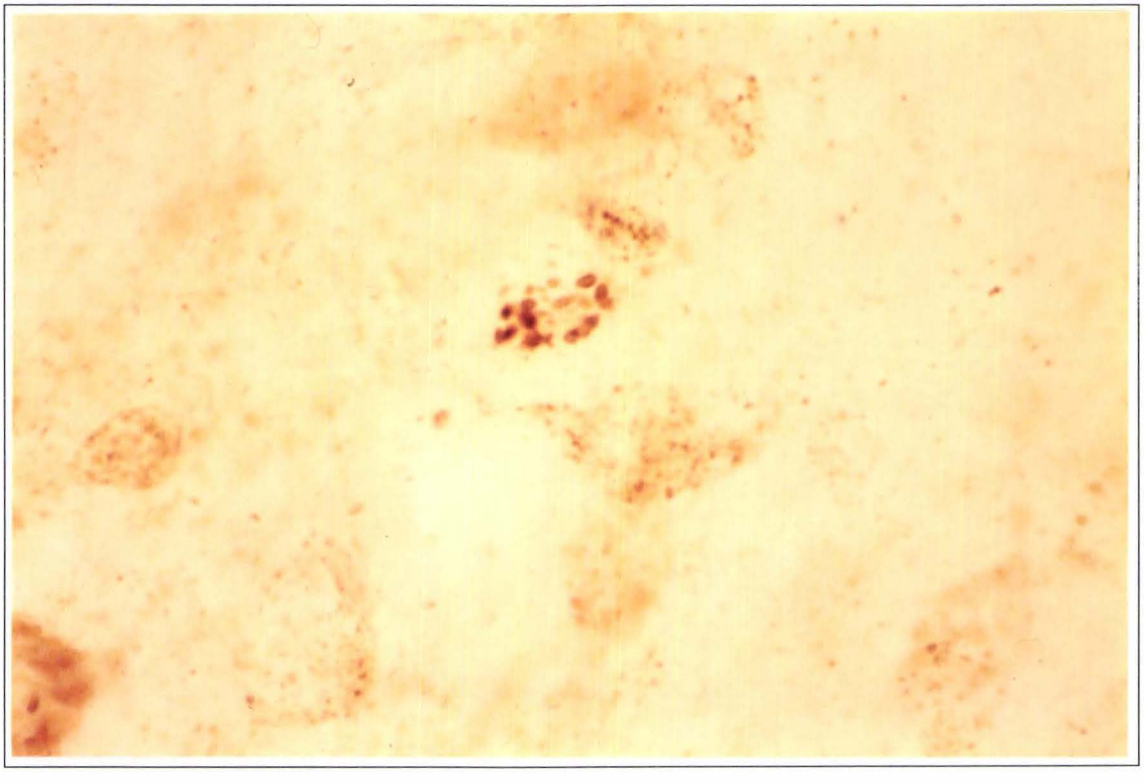


Figure 7-8: *Cerebral Cortex from a Sheep Affected with Ceroid-lipofuscinosis Immunostained using Anti-mitochodrial Antibody (Chemicon International Inc, Temecula, California, U.S.A); Arrow Enlarged Mitochondria. Vibratome Section* x 250.



Immunostaining of Muscle and Ear Cartilage

Immunostaining for diagnostic purposes was investigated in lambs known to be affected with ceroid-lipofuscinosis, and an equal number of unaffected control animals (Table 7-3). Strong positive staining of muscle and ear cartilage occurred in all affected lambs at 14 months of age (Figures 7-4 and 7-5) but was not observed in the preclinical state in lambs aged 2 $^1/_2$ months. In muscle fibres and in ear cartilage, immunostaining of structures was observed that were not identifiable by histochemistry or fluorescence microscopy as typical storage cytosomes. Diagnosis of ceroid-lipofuscinosis from muscle biopsy by histological staining with Sudan black, or fluorescence microscopy was possible in lambs at 14 months of age but diagnosis by immunostaining was easier as the distinction in staining patterns was clearly defined. Diagnosis by histochemical or fluorescence microscopy was not possible with ear cartilage samples.

Table 7-3. *Diagnosis of Ceroid-lipofuscinosis in Lambs by Immunostaining of Muscle and Ear Cartilage Biopsies at 2 $^1/_2$ and 14 Months of Age.*

Tissue	Age			
	2 $^1/_2$ months		14 months	
	OCL	Normal	OCL	Normal
Muscle	0/10*	0/14	10/10	9/9
Ear cartilage	0/10	0/14	10/10	10/10

Key

OCL	Lambs diagnosed with ceroid-lipofuscinosis by histopathology of brain biopsies.
Normal	Lambs shown to be phenotypically normal by histopathology of brain biopsies.
*	Number diagnosed with the correct phenotype/number examined by immunocytochemistry.

Immunostaining of a muscle biopsy from a child suspected as having late infantile Batten disease gave positive staining equivalent to that in affected lambs (Figure 7-9). However, in this case there was no advantage over histochemistry or fluorescence microscopy of the muscle biopsy. Immunostaining in a skin biopsy from the same child was also noted (Figure 7-10). The diagnosis was confirmed by electron microscopy of the muscle biopsy which showed curvilinear bodies typical of the late infantile form of disease (Figure 7-11). Histochemistry, fluorescence microscopy and immunostaining of a 3 year old clinically unaffected sibling did not demonstrate storage cytosomes. In view of the age factor demonstrated in lambs, caution with interpretation of this observation is necessary at this stage.

Immunostaining at the Ultrastructural Level

Immunogold staining of storage bodies occurred with all the antibodies to subunit c, antibodies to synthetic NH_2 terminal peptide of subunit c and with the antibody raised to the hydrophilic loop region of subunit c. This staining was seen in a variety of ovine tissues including cerebral cortex, cerebellum, heart muscle, pancreas (Figure 7-12a) and skeletal muscle (Figure 7-12b). A higher density background scattering of gold particles occurred with anti-NH_2-terminal peptide antibody from Dr. D.N. Palmer, with the anti-hydrophilic loop region of subunit c and with the anti DCCD-binding proteolipid antibody than with RabA antibody or the anti-NH_2-terminal peptide antibody from Dr. E. Kominami. No specific staining was observed in mitochondria or in any other cellular compartments or components using these antibodies.

Ultrastructural Immunostaining of Fibroblasts

The fixation protocol which gave the best results for the fibroblasts involved removal of the fibroblasts from the culture flask prior to incubation in the fixative solution. This method resulted in the most efficient harvest of fibroblasts, despite the extra steps involved. Apparent immunogold staining of storage body-like structures was suggested in a small proportion of the fibroblasts from affected sheep but this staining was equivocal (Figure 7-13). There was no specific labelling of mitochondria or labelling in close association with mitochondria in either affected or normal sheep.

Figure 7-9: *Muscle Biopsy from a Human Patient with Late Infantile Batten Disease Immunostained using Subunit c Antiserum (RabA); Paraffin Section* x 250. *Haematoxylin Counterstain.*

Figure 7-10: *Skin Biopsy from a Human Patient with Late Infantile Batten Disease Immunostained using Subunit c Antiserum (RabA); Paraffin Section* x 250. *Haematoxylin Counterstain.*

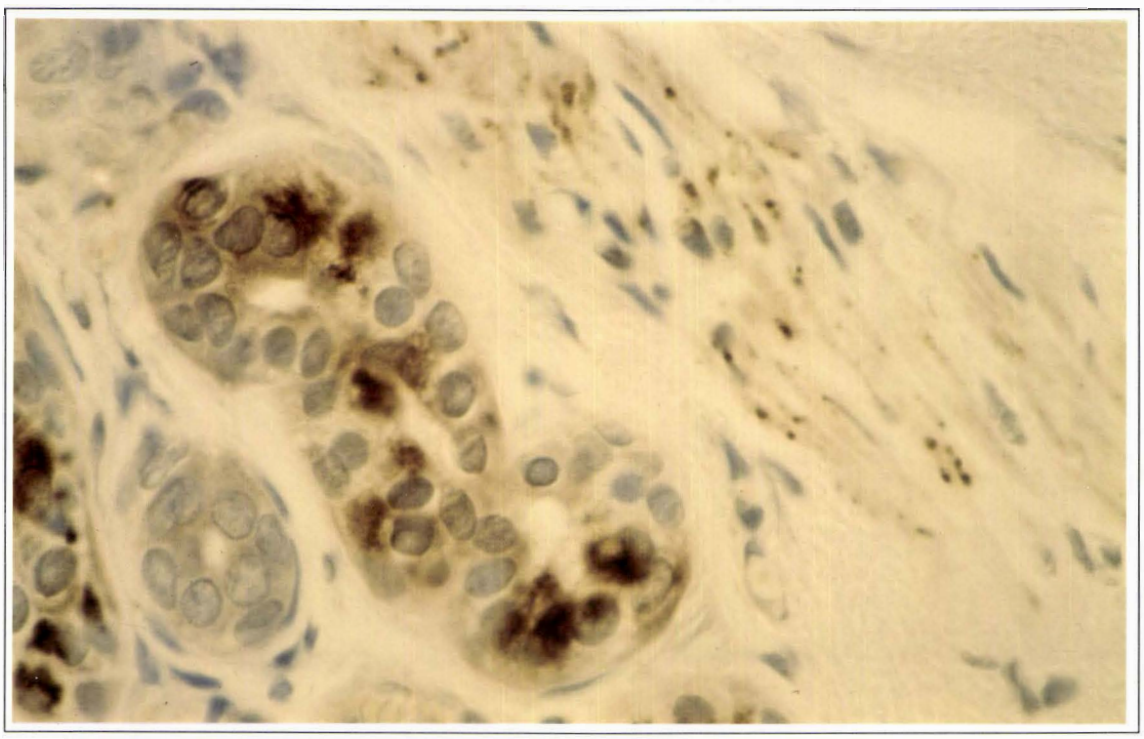


Figure 7-11: *Storage Cytosome Containing Curvilinear Profiles in Muscle from a Human Patient with Late Infantile Batten Disease;* EM x 42,400.

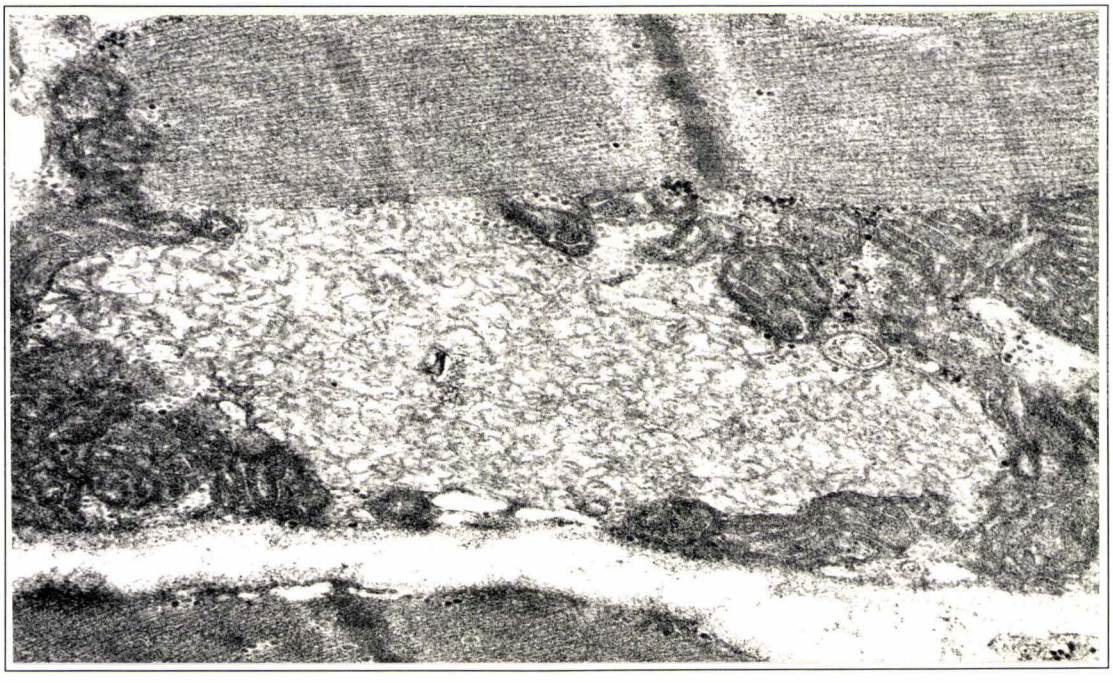
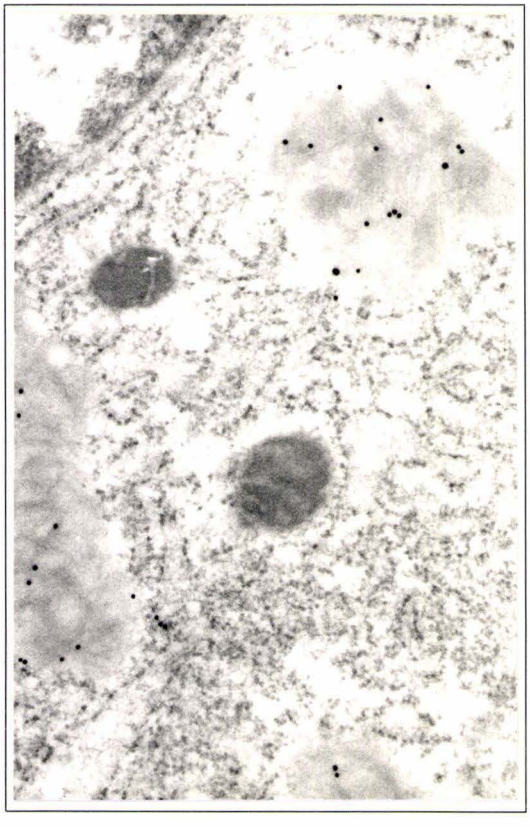


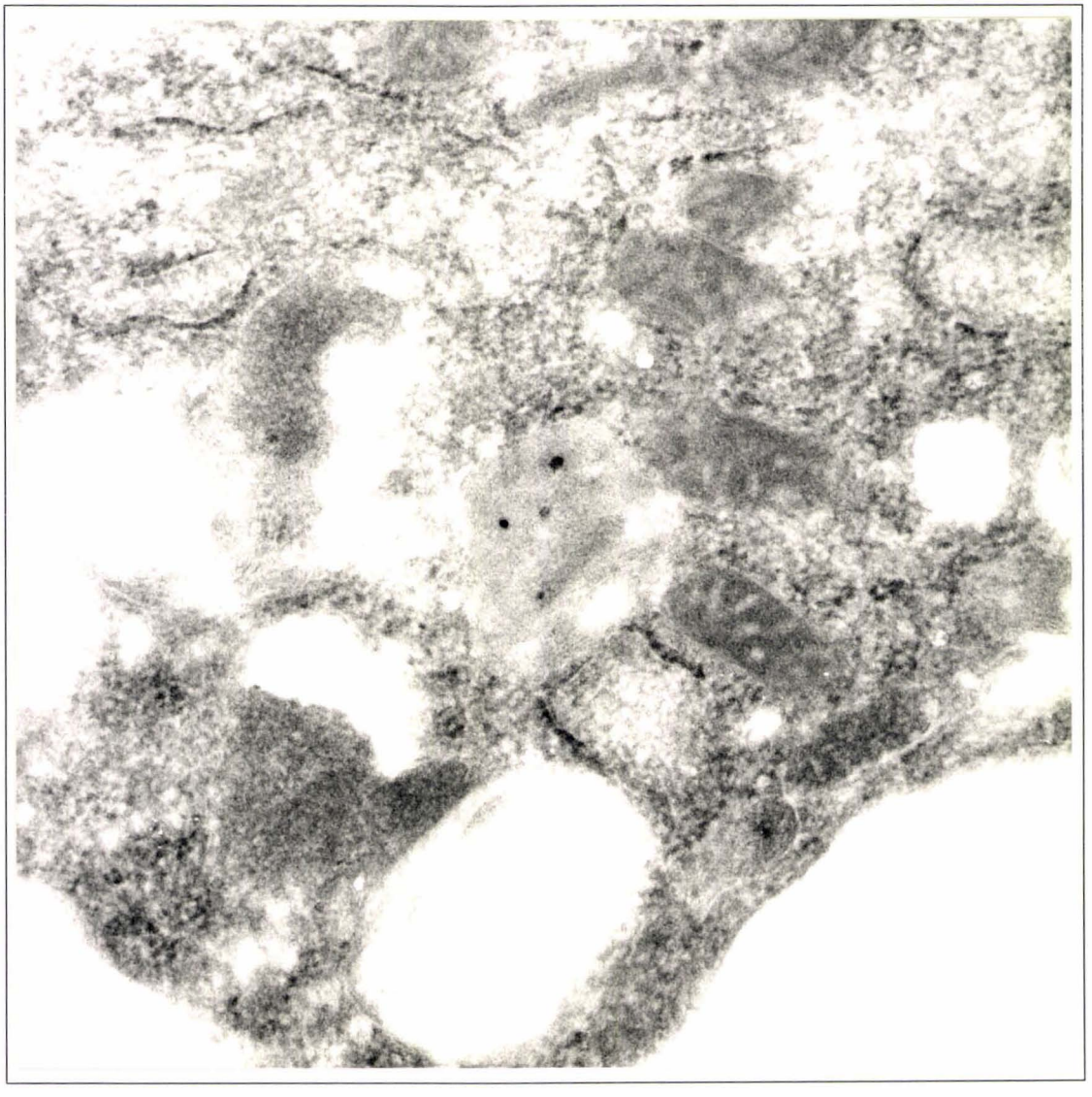
Figure 7-12: *Immunogold (15 nm) labelling of Storage Bodies in (a) Pancreas and (b) Skeletal Muscle of a Sheep Affected with Ceroid-lipofuscinosis using Subunit c Antiserum;* EM x 36,400.

(a)



(b)

Figure 7-13: *Apparent Immunogold Labelling of a Storage Body-like Structure in a Cultured Fibroblast from an Affected Sheep;*
EM x 76,500.



Discussion

The current study has proved that it is possible to produce high titre antibodies to subunit c of mitochondrial ATP synthase in rabbits using the liposomal RIBI system. Adjuvants, such as Freund's, have traditionally been formulated to delay the clearance of water soluble peptides long enough to enable antigen recognition to take place. In contrast, a liposomal system causes hydrophobic proteins like subunit c to be solubilised for long enough to enable antigen recognition to take place (Alving 1991). The resulting lack of activity from protocols using Freund's adjuvant suggests that antigen aggregation and lack of recognition is a problem which can frustrate attempts to raise antibodies to proteins like subunit c.

The specificity of antisera raised to subunit c proteolipid isolated from sheep liver storage bodies (RabA) was demonstrated on Western blots. This antisera was shown to compare favourably with antisera raised against synthetic peptides from the N-terminal portion of subunit c (Hall *et al* 1991b, Kominami *et al* 1992). A protein band at 3,500 MW was stained by RabA antisera on Western blots of protein from storage material, isolated subunit c proteolipid from storage material and subunit c from normal mitochondria. This suggests that the same antigen is present in the protein from storage bodies and in the mitochondrial protein.

Antibodies to subunit c were used for immunocytochemistry to detect subunit c storage in tissues from human and animal patients with various forms of ceroid-lipofuscinosis. The most critical singular determinant for successful immunostaining was fixation for less than 24 hours. Phosphate buffer immersion following formalin fixation improved immunostaining intensity. This can probably be attributed to crosslinking of the antigenic site proteins which may be partially reversed by the phosphate buffer immersion. The faint intensity of immunocytochemical staining in the canine, Devon cattle and Swedish lamb forms of the disease where the fixation regimes were unknown may be disease-related epitope differences (see below), due to prolonged fixation or due to epitope masking by other proteins which may also be present.

Polyclonal antibodies against subunit c have also been raised in rabbits by Hall *et al* (1991b) using storage material from human late infantile Batten disease, pancreas from a case of ovine ceroid-lipofuscinosis and a synthetic peptide corresponding to residues 36-48 of the subunit c sequence coupled via bis-diazobenzidine to ovalbumin. These antibodies were used for immunocytochemistry at the light microscopy level on tissues from late infantile,

juvenile and Kuf forms of Batten disease (Hall *et al* 1991a, b, Lake and Hall 1993). No antibody binding was reported to occur in tissues from normal patients. Comparison of the reported results from immunostaining with these antibodies, with RabA antibody, shows comparable staining intensity. However, the antibodies raised by Hall *et al* (1991b) reportedly stain late infantile and juvenile storage bodies irrespectively of the fixation conditions of the original tissue sample (Lake and Hall 1993). The specific epitope recognised by RabA may be particularly susceptible to alteration during fixation or alternatively, the epitopic sites in the sheep storage material may be sensitive to overfixation.

Different epitopes may be exposed in the different forms of ceroid-lipofuscinosis in humans and animals. This suggestion is supported by the different fixation susceptibilities (see above) and the differential staining intensity of storage bodies with different antibodies in different diseases. The whole storage cytosomes are consistently stained with anti subunit c antibodies in neurons in the cerebral cortex of a patient with late infantile human Batten disease. In contrast in the ovine form of the disease only the periphery of the storage bodies in neurons may be stained. Additionally, storage bodies from a patient with Kuf disease exhibited enhanced staining with synthetic NH_2 terminal peptide of subunit c antibody compared with whole subunit c antibody, whereas the reverse occurred in late infantile human and ovine Batten disease. Electron microscopy has revealed wide variability in the ultrastructure of storage bodies. This is evident between different forms of Batten disease and within different tissues within the same form of the disease. These differences are likely to be manifestations of different tertiary structures of the stored material; hence different epitopes are likely to be exposed at the surface of the storage bodies.

Immunocytochemical studies have indicated apparent accumulation of subunit c in neurons in sections from patients with other neurological disorders such as Sanfilipo disease, (mucopolysaccharidosis type IIIA) (Lake and Hall 1993, Kida *et al* 1993), Niemann Pick type C, mucopolysaccharidoses types I, II, and IIIA, polysulphatase deficiency, mucolipidosis I, sialidosis, Niemann-Pick type A and in GM_1 and GM_2 gangliosidosis (Elleder pers. comm.). The significance of these reports remains to be determined.

Subunit c antibodies detect storage material within some muscle fibres and in ear cartilage chondrocytes which the traditional histological stains (Sudan black, PAS and LFB) and fluorescence microscopy do not identify. This material may be an "early" form of storage body. Staining of this material illustrates the enhanced sensitivity of antibody staining over traditional histological stains.

Late infantile human storage bodies were stained with antisera to human β-hexosaminidase. The positive staining of these storage bodies confirms that storage is lysosomal. No reaction was observed with ovine and canine sections possibly because of species-specific epitope differences in the β-hexosaminidase enzyme.

Ultrastructural immunocytochemistry shows clear immunogold labelling of storage bodies but does not show any evidence of subunit c accumulation in mitochondria or in any other cellular organelles. Immunogold labelling of cultured fibroblasts suggested that storage bodies were labelled but these results were equivocal. Only a few storage body-like structures were seen in the cultured fibroblasts. This is not surprising given that the observed incidence of storage bodies will depend on the initial load combined with additional protein accumulation occurring during culture but will be diluted by rapid cell division. Fibroblasts multiply exponentially during the growth phase in culture and as subunit c appears to turnover only every 4-6 days (Grisolia *et al* 1985) the dilution effect for storage cytosomes can be expected to be considerable during the culture of fibroblasts. Tertiary lysosome-like structures may also form in cultured fibroblasts as a result of conditions during cell culture (Joshi *et al* 1989). These structures may be confused with storage bodies. The small size and hence low intensity of immunogold labelling also contributes to the difficulty in clearly identifying storage bodies in cultured fibroblasts. This is frustrating as the efficacy of using fibroblasts for metabolic studies in Batten disease would be enhanced by a reliable phenotype indicator of genotype.

Immunocytochemical staining using an antibody to subunit c of mitochondrial ATP synthase is a useful adjunct to other methods of diagnosis for some forms of Batten disease when using muscle biopsy samples in humans and sheep, and ear cartilage biopsy in sheep. Muscle biopsy appears to be an inadequately explored method of diagnosis of Batten disease, irrespective of whether histochemistry, fluorescence microscopy, electron microscopy or immunostaining is used.

Summary

High titre antibodies have been raised to subunit c of mitochondrial ATP synthase using the RIBI liposomal adjuvant system. Immunocytochemical staining has been carried out at the light and electron microscopy levels on tissues from animal and human forms of Batten disease. Strong immunocytochemical staining of storage bodies occurs in developed

disease but not in the preclinical stage in sheep, hence this technique has limitations as a preclinical diagnostic test. Immunocytochemical staining patterns of storage bodies differs in different forms of Batten disease, hence different epitopes appear to be exposed. Different antibodies raised to subunit c appear to differ in their sensitivity to epitope alteration by tissue fixation. Antibodies against subunit c also stain storage cytosomes at the ultrastructural level using the immunogold technique.

Chapter Eight

Summary and General Discussion

This thesis reviewed the current understanding of the diseases known as the ceroid-lipofuscinoses (Batten disease). Considerable progress has been made in some areas but in other respects these diseases remain almost as enigmatic today as when they were defined in the late 1960's by Zeman and colleagues (Zeman and Dyken 1969, Zeman 1970, Zeman *et al* 1970). For many years a defect in lipid metabolism was postulated as having a central role in the pathogenesis of the ceroid-lipofuscinoses. This was on the basis of histological staining, fluorescence properties and theories evolving from fatty acid metabolism studies. However, conclusive evidence to support an abnormality of lipid metabolism has not been forthcoming despite many years of intensive research.

Garrod (1908) enunciated the principle that inborn errors of metabolism should be reflected by elevation of the deficient enzyme's substrate or its derivatives. Following this principle, the discovery that subunit c of mitochondrial ATP synthase was the major stored component in some forms of Batten disease was a major breakthrough (Palmer *et al* 1988, 1990, 1992, Fearnley *et al* 1990). Identification of this protein with its extreme hydrophobicity provided an explanation for the ultrastructural appearance and physical properties of storage bodies and their lipid-like histological behaviour. Discovery of subunit c storage appeared a possible uniting factor for at least some of this heterogeneous group of diseases. Subunit c is not stored in the infantile human form of ceroid-lipofuscinosis (Palmer *et al* 1990, 1992 and Tyynelä *et al* 1993) and is found in only trace amounts in miniature Schnauzer dogs affected with a form of the disease (Jolly and Palmer 1995, Palmer *et al* unpublished). Analyses of storage material from the infantile human disease revealed that the sphingolipid activator proteins A and D are stored (Tyynelä *et al* 1993). Identification of the storage of different hydrophobic proteins within forms of the disease classified as ceroid-lipofuscinosis reflects the fact that histological and clinical similarities do not necessarily reflect biochemical homogeneity.

The studies which have been described within this thesis pertain to the prototype subunit c storage disease that was described in sheep. Clinically-based studies included staging of the disease and the results of studies involving early diagnosis by brain biopsy. It was concluded that brain biopsy is a safe and reliable method for early diagnosis of ceroid-lipofuscinosis in 2 $^{1}/_{2}$ month old lambs. In contrast, a clear distinction between normal and affected lambs could only be made comparatively late in the disease course at 9-12 months by clinical examination. However, as the disease progressed the combination of subjective neurological tests into a "neurological score" allowed clinical staging. This was used to monitor the efficacy of treatment of ceroid-lipofuscinosis by foetal haematopoietic cell transplantation (see below) and would be suitable as a means of assessing other treatment regimes.

Storage cytosomes in ceroid-lipofuscinosis show autofluorescence. This observation has lead to the assumption that a significant fluorophore of pathogenic significance is present. Studies on the fluorescence of storage bodies were carried out and storage body-like structures were reconstituted from isolated components. It was concluded that the fluorescence of storage bodies was attributable to the tertiary structure of protein in its particular environment and that no other significant fluorophore was present.

Bone marrow transplantation has proved a successful cure for several inherited metabolic storage disorders, particularly where the substrate is water soluble (see chapter two). Ceroid-lipofuscinosis is a disease for which there is no cure and it has a relentless, eventually tragic, progression. There has been interest and hope that bone marrow transplantation may be efficacious in this disease. However due to the complexity, seriousness and uncertainty regarding the outcome of this procedure it is desirable to assess its effect in an animal model. Bone marrow transplantation therapy has been attempted in 4-6 month old English setter dogs with ceroid-lipofuscinosis using heterozygous donor marrow (Deeg *et al* 1989, 1990). There was no indication that this treatment was beneficial. Similarly, it has been reported that liver transplantation has been carried out in dogs (Koppang 1993) without any indication of a beneficial effect but no details have been published. It can be argued in these instances that in 4-6 month old recipients the disease may already be too advanced to be altered by transplantation and that heterozygous donors may not provide optimal enzyme activity levels. To circumvent these problems, haematopoietic cell transplantation was undertaken in foetal lambs with ceroid-lipofuscinosis (see chapter five). An average of 9% engraftment of donor cells was achieved but no clinical or pathological benefit was demonstrated in affected lambs. There are two possible reasons

why this was unsuccessful. Firstly the level of donor cell engraftment may be too low and secondly, this may not be a disease which is amenable to treatment by metabolic collaboration by the transplanted cells.

The level of any enzyme present in normal tissues is well in excess of the basic minimum level required for substrate metabolism. The level of enzyme replacement after a 9% haematopoietic cell engraftment from a normal donor, performed very early in life, could possibly be expected to influence the course of the disease. Residual enzyme activities much lower than 9% have been found in a number of mild cases of inherited enzyme deficiencies and levels close to this have been found in apparently healthy individuals (Conzelmann and Sandhoff 1983/84).

Since the above studies, bone marrow transplantation has been attempted, apparently unsuccessfully, in a child with late infantile Batten disease (Lake *et al* 1995, News media reports). Further transplantation therapy in animal models or human patients should be preceded by studies to determine if metabolic collaboration between cells is possible in Batten disease. These studies have been traditionally carried out with cultured fibroblasts but in Batten disease few storage body inclusions are seen in fibroblasts. It has been suggested that these cells lose their storage body inclusions (Wolfe *et al* 1988b) and their ability to accumulate subunit c in long-term culture (Kida *et al* 1993). However, short term fibroblast cultures from late infantile Batten disease patients have been used successfully for metabolic studies by Ezaki *et al* (1993, 1995) and may thus be suitable for short term quantitative studies.

In late infantile Batten disease, lymphocytes consistently display storage in the form of curvilinear profiles. Lake *et al* (1995) have attempted co-culture experiments using normal lymphocytes with those from a patient with preclinical late infantile Batten disease. Inclusion bodies remained unaltered in co-cultured lymphocytes up to and including the 6th day of culture, providing no ultrastructural evidence of metabolic collaboration. The culture period may have been too short to observe any benefit and minor dissolution of storage bodies may not have been detected. Alternatively, it has been suggested that in ceroid-lipofuscinosis subunit c is complexed with lipids in a paracrystalline tertiary structure which may be resistant to the effect of any cellular enzymes (Jolly *et al* 1988, Jolly 1995). Therefore a lack of observed storage cytosome dissolution may not necessarily indicate that the primary metabolic defect has not been overcome. There remains a need for longer term co-culture studies with a means of measuring the level of storage product accurately. The

quantitative studies utilising an anti-subunit c antibody currently being evaluated and discussed below, may be useful for this. Neuronal cell cultures have been taken from the brains of English setters with ceroid-lipofuscinosis (Dunn *et al* 1994). These cells are reported to develop storage bodies within 3 weeks of culture and could be suitable for such metabolic collaboration studies.

A form of ceroid-lipofuscinosis has been reported in the *mnd* mouse in which subunit c of both ATP synthase and the related vacuolar ATPase accumulate (Faust *et al* 1994). Chimeric mice created by fusing normal and *mnd* embryonic cells developed populations of adjacent cells with and without storage bodies, indicating a lack of metabolic collaboration (Messer *et al* 1995).

There has been no evidence from the experiments reported and discussed above that metabolic collaboration occurs between cells in any of the human or animal forms of Batten disease investigated thus far. Additionally there has been no evidence that bone marrow or organ transplantation provides any beneficial effect in Batten disease. At this stage the prospects for bone marrow transplantation therapy in the forms of Batten disease where subunit c accumulates do not look promising but further experiments are warranted.

A major area of research was the development and utilisation of antibodies to subunit c of mitochondrial ATP synthase. Subunit c is a hydrophobic protein which is highly conserved between species. This factor combined with the general insolubility of subunit c leads to problems presenting the protein to the immune system. For these reasons antibodies to subunit c are difficult to produce. Various adjuvants and inoculation systems were tried but only that using a liposomal adjuvant system (RIBI) lead to the development of a high titre antibody to subunit c (RabA).

In addition to the use of RabA antibody for the studies described, it has been supplied to more than 20 laboratories around the world for research purposes. This and other antibodies were used for immunocytochemistry at the light and electron microscopy levels for diagnostic purposes and subcellular localisation studies. Successful immunocytochemical staining depended on light initial fixation of the tissues. This is reportedly not the case for another antibody to subunit c where initial fixation appears irrelevant at least in human cases (Lake and Hall 1993). RabA and other subunit c antibodies were effective in staining storage bodies in tissues *in situ* later in the disease course but not in the preclinical form. For this reason the use of this technique for diagnosis appears to be limited.

Antibodies from other sources were also used for immunocytochemistry. Different immunocytochemical staining patterns were seen at the light microscopic level with the different antibodies in different forms of the disease studied and between different tissue and cell types within the same form. It was concluded that this was due to exposure of different epitopes. Such is to be expected given the variety of multilamellar arrays exhibited ultrastructurally in the various forms of the disease. This probably reflects the different tertiary structure of protein(s) within the different arrays within storage bodies. Similar immunogold staining of storage bodies was noted ultrastructurally when any of the antibodies to subunit c or to the NH_2 terminal peptide of subunit c were used. No other cellular compartments or components were stained with these antibodies.

The solubility of subunit c in chloroform/methanol/ammonium acetate provides a method of extraction and semi-purification of this protein from tissues. Few other proteins, with the major exception of the proteolipid of myelin, are similarly extracted. This extraction technique when combined with a method of measuring extracted protein enables quantitative detection of subunit c accumulation. Unfortunately traditional methods such as the Lowry protein assay are unreliable with this protein (Sebald and Hoppe 1981). Detection methods based on an immunological reaction of subunit c with a specific antibody are currently being evaluated in this laboratory. They include enhanced chemiluminescence/autoradiography (see chapters three and five) and a modified ELISA technique.

There are many inherited lysosomal storage diseases of human beings (Neufeld 1991). These reflect a variety of catabolic defects but none are associated with defective catabolism of protein, as could be expected to occur by chance. Lysosomal protein catabolism is carried out by proteases which are peptide site specific rather than protein specific. Although there is only a limited number of these proteases, collectively they break down a myriad of proteins and overlapping activities can probably compensate for specific deficiencies. A number of different forms of ceroid-lipofuscinosis specifically accumulate the protein subunit c. These forms exhibit differences in the ultrastructure of storage bodies and have a different genetic basis. This diversity suggests that the respective defects vary in the different forms. It has been suggested that there may be a specific turnover pathway for subunit c (Palmer *et al* 1992, 1995) but because of the relative non-specificity of the mechanism for protein breakdown, it appears unlikely that the ceroid-lipofuscinoses result from the deficiency of a protease (Jolly 1995). However, the deficiency of, or defect in, an activating or transport protein cannot be discounted.

The pathological findings in the ceroid-lipofuscinoses of significant neuronal loss in the cerebral cortex and other selective brain regions have long indicated that these are neurodegenerative diseases, as well as storage diseases. Furthermore, it has been suspected that this neurodegeneration can be attributed to the disease process, rather than the overloading of cells by residual bodies (Zeman and Siakotos 1973). It has been proposed by Walkley *et al* (1995) that Batten disease may be closer to Alzheimer, Huntington and Parkinson diseases than to the classical storage diseases due to hydrolase deficiencies where neuronal cell death does not usually occur to the same extent.

Mitochondrial dysfunction has been suggested as being associated with cell death in the major human neurodegenerative diseases (Wallace 1992) and hence it is an interesting possibility that Batten disease may involve mitochondrial dysfunction also. There is a close spatial relationship between mitochondria and storage bodies within cells (Zeman and Donahue 1963). Other factors which provide support for this hypothesis include the location of subunit c in the inner mitochondrial membrane; a report by Ezaki *et al* (1995) that delayed removal of subunit c appears to occur within mitochondria; the identification of a small subset of mitochondrial proteins which appear to accumulate with subunit c (Moroni-Rawson *et al* 1995) and the observation by Walkley *et al* (1995) that structurally abnormal mitochondria are present in GABAergic cell populations in canine and ovine ceroid-lipofuscinosis. These were also observed in the present study (see chapter seven).

Studies utilising two dimensional PAGE of ovine liver storage body proteins have shown that a subset of mitochondrial proteins accumulate with subunit c in storage bodies in small amounts but relatively in excess of that in liver mitochondria (Moroni-Rawson *et al* 1995). Most other mitochondrial proteins could not be detected in storage bodies, so it did not appear that the accumulating proteins were present due to mitochondrial contamination of the preparations. Similar analysis of extramitochondrial proteins showed greater than 10-20 fold accumulation of ferritin light chains in microsomes, partial loss of a putative lysosomal protein, and decreased senescence marker protein in the cytosolic fraction from ovine ceroid-lipofuscinosis. The basis and significance of these differences is unknown.

Debate has ranged over the cause of selective cell death in Batten disease. Recently, Boustany (pers. comm.) and Lane *et al* (1995) have proposed that the cause of selective cell death in some forms of ceroid-lipofuscinosis is by apoptosis (also called programmed cell death) and that increased expression of the oncogene Bcl-2 can be demonstrated. The Bcl-2 oncogene is an inner mitochondrial membrane protein associated with inhibition of

apotosis (Hockenberry *et al* 1990). This protein is reportedly enriched within lipofuscin and autophagic vacuoles of neurons, glial and vascular cells in aged brain and in brains from patients with neurodegenerative diseases, Alzheimer disease, amylotrophic lateral sclerosis and in a form of prionic dementia (Migheli *et al* 1994). This raises the possibility that the observed Bcl-2 overexpression (up regulation) in Batten disease could be a cellular attempt at preventing apotosis; alternatively it may be an additional epiphenomenon resulting from perturbation of the inner mitochondrial membrane. Apoptotic cell death occurs in a variety of forms of retinal degeneration which are not dissimilar to those in the ceroid-lipofuscinoses (Lolley 1994). No single biochemical event has been demonstrated in every instance of apoptosis, hence there is disagreement regarding events in the process of apoptotic cell death. Whatever the pathogenesis of cell death in ceroid-lipofuscinosis, either necrosis or apoptosis, both may be mediated by mitochondrial dysfunction.

Ultrastructural, freeze-fracture and powder X-ray-diffraction studies have shown that storage bodies exhibit a complex, organised, three-dimensional structure suggestive of a paracrystalline array of protein complexed with lipids (Jolly *et al* 1988, Jolly 1995). Subunit c accumulation within cells may not necessarily reflect the inability to breakdown protein *per se* but may reflect an inability to catabolise it within a particular environment. This phenomenon has been reported to occur in *Escherichia coli* where high level expression of eukaryotic genes may result in proteinaceous aggregates (Kane and Hartley 1988). Cheng *et al* (1981) provide evidence that some of these aggregates may not be catabolised by intracellular proteases, even though these proteases are competent at digesting the proteins under different cellular circumstances.

All cell membranes contain phospholipid bilayers which are fluid at normal body temperature. Phospholipid molecules in general can move freely within the membrane but require special "flip-flop" proteins to move between the different bilayers. However, lipid molecules surrounding proteins are strongly immobilized within membranes (lipid boundary layer immobilisation) and lipid molecules between closely orientated proteins demonstrate strong perturbation of lipid bilayer fluidity (bilayer perturbation by protein packing) (Marsh *et al* 1978).

Subunit c is a hydrophobic protein with a propensity for aggregation with itself or lipids. Aggregation could theoretically occur at any point in the turnover pathway but hypothetically it is most likely to occur during the initial disassembly of the F_o complex, where multiple copies of subunit c are present. Regardless of the cellular location of subunit c catabolism

it is possible that any perturbation of the inner mitochondrial membrane environment could affect the disassembly of subunit c from this environment. The inner mitochondrial membrane contains 76% by weight protein (Darnell *et al* 1990), hence the lipid-protein ratio lies in the range for which strong perturbation of the lipid bilayer is observed, suggesting boundary lipid and perturbation of fluidity effects may be important. Any consideration of the disassembly in part, or whole, of the F_o complex as an initial step in the catabolic pathway of subunit c should include the boundary lipids, i.e. it should involve the "F_o complex domain". The first step in this disassembly process may not necessarily be mediated by a protease.

It has been demonstrated that storage material does not consist of products of lipid peroxidation (Palmer *et al* 1986a, b, 1989, 1990, Fearnley *et al* 1990) as was perceived for over two decades but the possibility that the ceroid-lipofuscinoses involve a defect in lipid metabolism cannot be entirely discounted. Polyunsaturated fatty acids are the fatty acids of greatest importance to many membrane functions, including the maintenance of normal membrane fluidity. Differences in certain phospholipids have been reported between normal individuals and patients with Batten disease but there is very little consistency in the literature. Svennerholm *et al* (1975, Svennerholm 1976) initially reported that the fatty acid profiles of the major phospholipids isolated from grey matter of patients with infantile ceroid-lipofuscinosis differed from those of normal controls. Pullarkat and colleagues (1982) reported abnormal grey matter phosphatidylserine in the infantile, late infantile, adult and "pigment variant" forms of the disease. Other phospholipids were reportedly normal.

In patients with juvenile ceroid-lipofuscinosis, abnormalities have been reported in circulating lipoproteins (Bennett *et al* 1988), in phospholipids and fatty acids in erythrocytes and platelets (Bennett *et al* 1990) and decreased lymphocyte membrane fluidity (Kohlschütter *et al* 1988). Photoreceptors in the retinal membrane are known to be enriched with polyunsaturated fatty acids, in particular docosahexaenoic acid (22:6 w3). This fatty acid has been reported to be decreased in the retina in English setter dogs with ceroid-lipofuscinosis (Reddy *et al* 1985). However, analyses comparing phospholipid fatty acids in brains of sheep with ceroid-lipofuscinosis with normal sheep failed to show any sign of a significant essential fatty acid abnormality (Palmer *et al* 1985). In comparing the analyses from human patients and sheep it should be noted that the human subjects had died of the disease whereas the sheep were euthanased at comparatively earlier ages. Additionally, human patients receive a wide spectrum of drug treatments necessary to control seizures and

improve quality of life and it is difficult to assess the effect these may have had on cell membranes. It is difficult to determine if changes in fatty acid profiles are of aetiological significance or rather are consequent to the degenerative changes peculiar to these diseases.

Dietary supplementation studies provide evidence to refute the lipid peroxidation hypothesis but provide support for a putative defect in lipid metabolism. Koppang (1987) fed affected English setter dogs a diet containing 20% polyunsaturated fatty acids and compared the disease course with that of dogs receiving antioxidant supplementation and those on a normal diet. The dogs receiving the polyunsaturated fatty acid supplementation had an increased life span and enhanced quality of life. Bennett *et al* (1988) reported an altered content of membrane fatty acids and increased membrane deformity following polyunsaturated fatty acid supplementation in patients with juvenile Batten disease. Further reports by Bennett *et al* (1993, 1994) of an experimental but uncontrolled treatment regime where juvenile Batten disease patients received polyunsaturated fatty acids supplementation for over 7 years, provides evidence suggestive of favourable modification of the disease course. Further controlled dietary supplementation trials and biochemical research needs to be carried out in this area.

Mitochondria in cells increase in numbers by the process of division. The inner mitochondrial membrane in new mitochondria grows by accretion of newly synthesised materials from the cytosol and the mitochondrion itself. It has been assumed that mitochondrial proteins are degraded by lysosomes because mitochondria are frequently seen inside autophagic vacuoles. However, it has been demonstrated that different mitochondrial proteins have different half-lives of either a few hours, or 4-6 days (reviewed by Grisolia *et al* 1985). Proteases have been shown to exist in the matrix of mitochondria (Desautels and Goldberg 1982, Watabe and Kimora 1985) and these are thought to be responsible for the turnover of proteins with a short half life. Very little is known about the mechanisms of turnover of longer half-life membrane-bound proteins. This turnover may involve autophagy, matrix enzymes, a combination of both, or a different method entirely. Lipsky and Pedersen (1981) also suggest that random breakdown of mitochondria appears to occur and that mitochondrial degradation may be more a function of positional differences such as proximity to a lysosome within the cell, rather than mitochondrial quality or size. Both the mitochondrial matrix and cellular lysosomes contain proteases which are able to catabolise protein molecules (Grisolia *et al* 1985, Chandler and Ballard 1988). Four possible schemata for subunit c breakdown can be postulated: *(a)* disassembly of the F_o complex and catabolism of subunit c may occur by mitochondrial matrix enzymes; *(b)* the F_o complex may be

disassembled in mitochondria and then subunit c transported out to be catabolised in lysosomes or within the cytoplasm; *(c)* subunit c could be turned over both in mitochondria and in lysosomes and *(d)* autophagy of senescent mitochondria, or senescent portions of mitochondria may occur and F_o complex disassembly and subunit c catabolism may occur within the lysosomal system. Further studies need to be carried out in this area to clarify where and how the turnover of subunit c is carried out.

It can be concluded that the ceroid-lipofuscinoses are a complex group of diseases, with complex systems underlying them. Despite priority funding and much research effort, progress on the elucidation of the pathogenesis and underlying biochemical basis of these diseases is slow. At least four of the human diseases are not alleic at the genetic level and the traditional approach to storage diseases where the stored metabolite is expected to reflect the primary biochemical basis does not appear as fruitful as originally expected. Metabolic collaboration does not appear to occur between normal cells and Batten disease affected cells implying that the disease may not be amenable to treatment by bone marrow transplantation. Elucidation of the turnover pathway of longer half-life membrane-bound proteins may provide some insight into its pathogenesis. Subcellular localisation and labelling studies may be useful as a tool in this process. Molecular biology through gene linkage and genetic mapping may eventually provide the biochemical answers sought in this disease. In the interim, direct cell biology studies hold promise for the development of improved diagnostic and therapeutic strategies for patients with ceroid-lipofuscinosis.

Bibliography

Adinolfi, M., Akle, C.A., McColl, I., Fensom, A.H., Tansley, L., Connolly, P., Hsi, B.-L., Faulk, W.P., Travers, P. and Bodmer, W.F. (1982): Expression of HLA antigens, β_2-microglobulin and enzymes by human amniotic epithelial cells. *Nature* **295**:325-327.

Akle, C.A., Adinolfi, M., Welsh, K.I., Leibowitz, S. and McColl, I. (1981): Immunogenicity of human amniotic epithelial cells after transplantation into volunteers. *Lancet* **ii**:1003-1005.

Allison, A.C. and Gregoriadis, G. (1974): Liposomes as immunological adjuvants. *Nature* **252**:252

Alroy, J., Schelling, S.H., Thalhammer, J.G., Raghavan, S.S., Natowicz, M.R., Prence, E.M. and Orgad, U. (1992): Adult onset lysosomal storage disease in a Tibetan terrier: Clinical, morphological and biochemical studies. *Acta Neuropathol.* **84**:658-663.

Alving, C.R. (1991): Liposomes as carriers of antigens and adjuvants. *J. Immunol. M.* **140**:1-13.

Amzel, L.M. and Pedersen, P.L. (1983): Proton ATPases: Structure and mechanism. *Ann. Rev. Biochem.* **52**:801-824.

Andermann, E., Jacob, J.C., Andermann, F., Carpenter, S., Wolfe, L. and Berkovic, S.F. (1988): The Newfoundland aggregate of neuronal ceroid-lipofuscinosis. *Am. J. Med. Genet., Suppl.* **5**:111-116.

Anderson, W.F. (1984): Prospects for human gene therapy. *Science* **226**:401-409.

Andersson, M., Appelkvist, E.L., Kristensson, K. and Dallner, G. (1987): Distribution of dolichol and dolichyl phosphate in human brain. *J. Neurochem.* **49**:685-691.

Appleby, E.C., Longstaffe, J.A. and Bell, F.R. (1982): Ceroid-lipofuscinosis in two Saluki dogs. *J. Comp. Pathol.* **92**:375-380.

Armstrong, D., Dimmitt, S., Grider, L., VanWormer, D. and Austin, J. (1973): Deficient leukocyte peroxidase activity in the Batten-Spielmeyer-Vogt syndrome. *Am. Neurol. Assoc. Trans.* **98**:3-7.

Armstrong, D. (1982): Peroxidase activity in human and canine ceroid-lipofuscinosis, with special emphasis on the eye. In *Ceroid-lipofuscinosis (Batten's disease).* (Eds. Armstrong, D., Koppang, N. and Rider, J.A.) pp.247-270. Elsevier Biomedical Press, Amsterdam.

Arnold, T.W., Faught, E., Falcon, J.C., Herrerea, G.A. and Linton, J. (1987): Adult onset neuronal neuronal ceroid lipofuscinosis (Kuf's disease) with autosomal dominant inheritance. *Neurology* **37**:138-139.

Barranger, J.A., Rapoport, S.I. and Brady, R.O. (1980): Access of enzymes to brain following osmotic alteration of the blood-brain barrier. *Birth Defects* **16**:195-205.

Barton, N.W., Furbish, F.S., Murray, G.J., Garfield, M. and Brady, R.O. (1990): Therapeutic response to intravenous infusions of glucocerebrosidase in a patient with Gaucher disease. *Proc. Natl. Acad. Sci. USA* **87**:1913-1916.

Barton, N.W., Brady, R.O., Dambrosia, J.M., Di Bisceglie, A.M., Doppelt, S.H., Hill, S.C., Mankin, H.J., Murray, G.J., Parker, R.I., Argoff, C.E., Grewall, R.P., Yu, K.-T. and collaborators (1991): Replacement therapy for inherited enzyme deficiency-Macrophage-targeted glucocerebrosidase for Gaucher's disease. *New Engl. J. Med.* **324**:1464-1470.

Bastedo, L., Sands, M.S., Lambert, D.T., Pisa, M.A., Birkenmeier, E. and Chang, P.L. (1994): Behavioural consequences of bone marrow transplantation in the treatment of murine mucopolysaccharidosis type VII. *J. Clin. Invest.* **94**:1180-1186.

Batten, F.E. (1903): Cerebral degeneration with symmetrical changes in the maculae in two members of a family. *Trans. Ophthalmol. Soc. UK.* **23**:386-390. Cited by Dyken, P.R. (1988): Reconsideration of the classification of the neuronal ceroid-lipofuscinoses. *Am. J. Med. Genet., Suppl.* **5**:69-84.

Batten, F.E. (1914): Family cerebral degeneration with macular change (so-called juvenile form of family amaurotic idiocy). *Quart. J. Med.* **7**:444-453. Cited by Zeman, W. and Dyken, P. (1969): Neuronal ceroid-lipofuscinosis (Batten's disease): Relationship to amaurotic family idiocy? *Pediatrics* **44**:570-583.

Bennett, M.J., Hosking G.P., Gayton, R., Thompson, G., Galloway, J.H. and Cartwright, I.J. (1988): Therapeutic modification of membrane lipid abnormalities in juvenile neuronal ceroid-lipofuscinosis (Batten disease). *Am. J. Med. Genet., Suppl.* **5**:275-281.

Bennett, M.J., Galloway, J.H., Cartwright, I.J., Gillis, W.S., Hosking, G.P. (1990): Decreased erythrocyte and platelet phospholipids and fatty acids in juvenile neuronal ceroid-lipofuscinosis (Batten's disease). *Neuropediatrics* **21**:202-205.

Bennett, M.J., Poirier, S.F., Chern, L., Gayton, A.R., Hosking, G.P., Le, N.-A., Majumdar, S. and Korchak, H.M. (1993): Juvenile neuronal ceroid-lipofuscinosis: Characterization of the dyslipoproteinaemia and demonstration of membrane phospholipid and phospholipid-dependent signal transduction abnormalities in cultured skin fibroblasts. *J. Inher. Metab. Dis.* **16**:308-311.

Bennett, M.J., Gayton, A.R., Rittey, C.D.C. and Hosking, G.P. (1994): Juvenile neuronal ceroid-lipofuscinosis: Developmental progress after supplementation with polyunsaturated fatty acids. *Dev. Med. Child. Neurol.* **36**:630-638.

Berkovic, S.F., Carpenter, S., Andermann, F., Andermann, E. and Wolfe, L.S. (1988): Kufs' disease: a critical reappraisal. *Brain* **111**:27-62.

Beutler, E., Dale, G.L., Guinto, E. and Kuhl, W. (1977): Enzyme replacement therapy in Gaucher's disease: Preliminary clinical trial of a new enzyme preparation. *Proc. Natl. Acad. Sci. USA* **74**:4620-4623.

Beutler, E. (1981): Enzyme replacement therapy. *TIBS* **6**:95-97.

Bichsel, P. and Vandevelde, M. (1982): Un casde lipofusinose céroide un berger de Yougoslavie. *Schweizer Archiv für Tierheilkunde* **124**:413-418.

Bielschowsky, M. (1913): Über spät-infantile familiäre amaurotische Idiotie mit Kleinhirnsymptomen. *Dtsch. Z. Nervenheilk* **50**:7-29. Cited by Dyken, P.R. (1988): Reconsideration of the classification of the neuronal ceroid-lipofuscinoses. *Am. J. Med. Genet., Suppl.* **5**:69-84.

Bienzle, D., Abrams-Ogg, A.C.G., Kruth, S.A., Ackland-Snow, J., Carter, R.F., Dick, J.E., Jacobs, R.M., Kamel-Reid, S. and Dubé, I.D. (1994): Gene transfer into hematopoietic stem cells: Long-term maintenance of *in vitro* activated progenitors without marrow ablation. *Proc. Natl. Acad. Sci. USA* **91**:350-354.

Bignami, A., Palladini, G. and Borne, P. (1969): Neuronal lipidosis with visceral involvement in an adult. *Acta Neuropathol.* **12**:50-61.

Birkenmeier, E.H., Barker, J.E., Vogler, C.A., Kyle, J.W., Sly, W.S., Gwynn, B., Levy, B. and Pegors, C. (1991): Increased life span and correction of metabolic defects in murine mucopolysaccharidosis type VII after syngeneic bone marrow transplantation. *Blood* **78**:3081-3092.

Blood, D.C., Radostits, O.M. and Henderson, J.A. (1983): *Veterinary Medicine. A textbook of the diseases of cattle, sheep, pigs, goats and horses.* Sixth edition. pp. 804-805. Baillière Tindall, London.

Boehme, H.D., Cottrell, J.C., Leonberg, S.C., and Zeman, W. (1971): A dominant form of neuronal ceroid-lipofuscinosis. *Brain* **94**:745-760. Cited by Zeman, W. (1976): The neuronal ceroid-lipofuscinoses. In *Progress in Neuropathology,* Volume III. (Ed. Zimmerman, H.M.). pp. 203-223. Grune and Stratton, New York.

Bou-Gharios, G., Adams, G., Pace, P. and Olsen, I. (1992): Long-term effects of bone marrow transplantation on lysosomal enzyme replacement in β-glucuronidase-deficient mice. *J. Inher. Metab. Dis.* **15**:899-910.

Boustany, R.-M. N., Alroy, J. and Kolodny, E. H. (1988): Clinical classification of neuronal ceroid-lipofuscinosis subtypes. *Am. J. Med. Genet., Suppl.* **5**:47-58.

Braak, H. and Goebel, H.H. (1978): Loss of pigment-laden stellate cells: A severe alteration of the isocortex in juvenile neuronal ceroid lipofuscinosis. *Acta Neuropathol.* **42**:53-57.

Braak, H. and Goebel, H.H. (1979): Pigmentoarchitectonic pathology of the isocortex in juvenile neuronal ceroid lipofuscinosis: Axonal enlargements in layer IIIab and cell loss in layer *V. Acta Neuropathol.* **46**:79-83.

Bradbury, M. (1979): *The Concept of a Blood-Brain Barrier.* pp. 308-310. John Wiley and Sons Ltd., New York.

Brady, R.O., Tallman, J.F., Johnson, W.G., Gal, A.E., Leahy, W.R., Quirk, J.M. and Dekaban, A.S. (1973): Replacement therapy for inherited enzyme deficiency. Use of purified ceramidetrihexosidase in Fabry's disease. *New Engl. J. Med.* **289**:9-14.

Brady, R.O., Pentchev, P.G., Gal, A.E., Hibbert, S.R. and Dekaban, A.S. (1974): Replacement therapy for inherited enzyme deficiency. Use of purified glucocerebrosidase in Gaucher's disease. *New Engl. J. Med.* **291**:989-993.

Brady, R.O., Pentchev, P.G. and Gal, A.E. (1975): Investigations in enzyme replacement therapy in lipid storage diseases. *Fed. Proc.* **34**:1310-1315.

Brady, R.O., Doppelt, S.H., Mankin, H.J., DiBisceglie A.M., Hill, S.C., Verderese, C.L., Graham, O.C., Argoff, C.E., Grewal, R.P. and Yu, Y.-T. (1990): Clinical effectiveness of enzyme replacement in Gaucher's disease. *Clin. Res.* **38**:457A.

Brewer, B.D. (1983): Neurologic disease of sheep and goats. *Vet. Clin. North Am. (Large Anim. Pract.)* **5**:677-700.

Bronson, R.T., Lake, B.D., Cook, S., Taylor, S. and Davisson, M.T. (1993): Motor neuron degeneration of mice is a model of neuronal ceroid lipofuscinosis (Batten's disease). *Ann. Neurol.* **33**:381-385.

Brown, N.J., Corner, D.B. and Dodgson, M.C.H. (1954): A second case in the same family of congenital familial cerebral lipoidosis resembling amaurotic family idiocy. *Arch. Dis. Childh.* **29**:48-54. Cited by Garborg, I., Torvik, A., Hals, J., Tangsrud, S.E. and Lindemann, R. (1987): Congenital neuronal ceroid lipofuscinosis. *Acta Pathol. Microbiol. Immunol. Scand.* **95**:119-125.

Brown, L.R., Lauterwein, J., Wüthrich, K. (1980): High-resolution ^{1}H-NMR studies of self-aggregation of melittin in aqueous solution. *Biochim. Biophys. Acta* **622**:231-244.

Broxmeyer, H.E., Kurtzberg, J., Gluckman, E., Auerbach, A.D., Douglas, G., Cooper, S., Falkenburg, J.H.F., Bard, J. and Boyse, E.A. (1991): Umbilical cord blood hematopoietic stem and repopulating cells in human clinical transplantation. *Blood Cells* **17**:330-337.

Callen, D.F., Baker, E., Lane, S., Nancarrow, J., Thompson, A., Whitmore, S.A., MacLennan, D.H., Berger, R., Cherif, D., Järvelä, I., Peltonen, L., Sutherland, G.R. and Gardiner, R.N. (1991): Regional mapping of the Batten disease locus (CLN3) to human chromosome 16p12. *Am. J. Hum. Genet.* **49**:1372-1377.

Chandler, C.S. and Ballard, F.J. (1988): Regulation of the breakdown rates of biotin-containing proteins in Swiss 3T3-L1 cells. *Biochem. J.* **251**:749-755.

Chen, R.F. (1990): Fluorescence of proteins and peptides. In *Practical Fluorescence.* (Ed. Guilbault, G.G.). Second edition. pp. 575-682. Marcel Dekker Inc., New York.

Cheng, Y.-S.E., Kwoh, D.Y., Kwoh, T.J., Soltvedt, B.C. and Zipser, D. (1981): Stabilization of a degradable protein by its overexpression in *Escherichia coli. Gene* **14**:121-130.

Chio, K.S. and Tappel, A.L. (1969a): Synthesis and characterization of the fluorescent products derived from malonaldehyde and amino acids. *Biochemistry* **8**:2821-2827.

Chio, K.S. and Tappel, A.L. (1969b): Inactivation of ribonuclease and other enzymes by peroxidizing lipids and by malonaldehyde. *Biochemistry* **8**:2827-2832.

Cho, D.-Y., Leipold, H.W. and Rudolph, R. (1986): Neuronal ceroidosis (ceroid-lipofuscinosis) in a blue heeler dog. *Acta Neuropathol.* **69**:161-164.

Clapp, D.W., Dumenco, L.L., Hatzoglou, M. and Gerson, S.L. (1991): Fetal liver hematopoietic stem cells as a target for in utero retroviral gene transfer. *Blood* **78**:1132-1139.

Collinson, I.R., Runswick, M.J., Buchanan, S.K., Fearnley, I.M., Skehel, J.M., van Raaij, M.J., Griffiths, D.E.and Walker, J.E. (1994): F_o membrane domain of ATP synthase from bovine heart mitochondria: Purification, subunit composition, and reconstitution with F_1-ATPase. *Biochemistry* **33**:7971-7978.

Constantinidis, J., Wisniewski, K.E. and Wisniewski, T.M. (1992): The adult and a new late adult forms of neuronal ceroid lipofuscinosis. *Acta Neuropathol.* **83**:461-468.

Conzelmann, E. and Sandhoff, K. (1983/84): Partial enzyme deficiencies: Residual activities and the development of neurological disorders. *Dev. Neurosci.* **6**:58-71.

Culling, C.F.A. (1974): *Handbook of Histopathological and Histochemical Techniques.* Third edition. pp. 262-272. Butterworths and Co. (Publishers) Ltd. London.

Culling, C.F.A., Allison, R.T. and Barr, W.T. (1985): *Cellular Pathology Technique.* Fourth edition. p. 264. Butterworths and Co. (Publishers) Ltd. London.

Culliton, B.J. (1990): Gene therapy begins. *Science* **249**:1372.

Cummings, J.F. and de Lahunta, A. (1977): An adult case of canine neuronal ceroid-lipofuscinosis. *Acta Neuropathol.* **39**:43-51.

Cummings, J.F., de Lahunta, A., Riis, R.C. and Loew, E.R. (1991): Neuropathologic changes in a young adult Tibetan terrier with subclinical ceroid-lipofuscinosis. *Prog. Vet. Neurol.* **1**:301-309.

Daniel, P.F., Sauls, D.L. and Boustany, R.-M.N. (1992): Evidence for processing of dolichol-linked oligosaccharides in patients with neuronal ceroid-lipofuscinosis. *Am. J. Med. Genet.* **42**:586-592.

Dalefield, R.R., Palmer, D.N. and Jolly, R.D. (1994): Lipofuscin and abnormalities in colloid in the equine thyroid gland in relation to age. *J. Comp. Pathol.* **111**:389-399.

Darnell, J., Lodish, H. and Baltimore, D. (1990): The plasma membrane. In *Molecular Cell Biology.* Second Edition. pp. 491-530. Scientific American Books, Inc., New York.

Dawson, G. and Glaser, P. T. (1988): Abnormal cathepsin B activity in Batten disease. *Am. J. Med. Genet., Suppl.* **5**:209-220.

Dawson, G., Kilkus, J. and Siakotos, A.N. (1993): Reduced phospholipase activity, peptide storage and the pathogenesis of canine neuronal ceroid-lipofuscinosis. *J. Inher. Metab. Dis.* **16**:330-334.

Dean, M.F., Muir, H. and Benson, P.F. (1973): Mobilization of glycosaminoglycans by plasma infusion in mucopolysaccharidosis type III - Two types of response. *Nat. New Biol.* **243**:143-146.

Dean, M.F., Muir, H., Benson, P.F., Button, L.R., Batchelor, J.R. and Bewick, M. (1975): Increased breakdown of glycosaminoglycans and the appearance of corrective enzyme after skin transplants in Hunter syndrome. *Nature* **257**:609-612.

Dean, M.F., Muir, H., Benson, P.F., Button, L.R., Boylston, A. and Mowbray, J. (1976): Enzyme replacement therapy by fibroblast transplantation in a case of Hunter syndrome. *Nature* **261**:323-325.

Dean, M.F., Stevens, R.L., Muir, H., Benson, P.F., Button, L.R., Anderson, R.L., Boylston, A. and Mowbray, J. (1979): Enzyme replacement therapy by fibroblast transplantation. Long-term biochemical study in three cases of Hunter's syndrome. *J. Clin. Invest.* **63**:138-145.

Dean, M.F., Muir, H., Benson, P.F. and Button, L.R. (1981): Enzyme replacement therapy by transplantation of HLA-compatible fibroblasts in Sanfilippo A syndrome. *Pediatr. Res.* **15**:959-963.

Deeg, H.J., Koppang, N., Shulman, H.M., Albrechtsen, D., Graham, T.C. and Storb, R. (1989): Allogeneic bone marrow transplantation for canine ceroid lipofuscinosis. *Transplant. Proc.* **21**:3082-3083.

Deeg, H.J., Shulman, H.M., Albrechtsen, D., Graham, T.C., Storb, R. and Koppang, N. (1990): Batten disease: failure of allogeneic bone marrow transplantation to arrest disease progression in a canine model. *Clin. Genet.* **37**:264-270.

DeLellis, R.A., Balogh, K., Merk, F.B., Chirife, A.M. (1972): Distinctive hepatic cell globules in adult alpha-1-antitrypsin deficiency. *Arch. Pathol.* **94**:308-316.

Desautels, M. and Goldberg, A.L. (1982): Liver mitochondria contain an ATP-dependent, vanadate-sensitive pathway for the degradation of proteins. *Proc. Natl. Acad. Sci. USA* **79**:1869-1873.

Desnick, R.J., Thorpe, S.R. and Fiddler, M.B. (1976): Towards enzyme therapy for lysosomal storage diseases. *Physiol. Rev.* **56**:57-99.

Dickson, L.R., Dopfmer, I., Dalefield, R.R., Graydon, R.J. and Jolly, R.D. (1989): A method of cerebro-cortical biopsy in lambs. *NZ Vet. J.* **37**:21-22.

Di Ferrante, N., Nichols, B.L., Donnelly, P.V., Neri, G., Hrgovcic, R. and Berglund, R.K. (1971): Induced degradation of glycosaminoglycans in Hurler's and Hunter's syndromes by plasma infusion. *Proc. Natl. Acad. Sci. USA* **68**:303-307.

Di Marco, P.N., Howell, J.McC. and Dorling, P.R. (1985): Bovine generalised glycogenosis type II: Uptake of lysosomal α-glucosidase by cultured skeletal muscle and reversal of glycogen accumulation. *FEBS Lett.* **190**:301-304.

Dobrenis, K., Joseph, A. and Rattazzi, M.C. (1992): Neuronal lysosomal enzyme replacement using fragment C of tetanus toxin. *Proc. Natl. Acad. Sci. USA* **89**:2297-2301.

Doering, L.C. and Chang, P.L. (1991): Expression of a novel gene product by transplants of genetically modified primary fibroblasts in the central nervous system. *J. Neurosci. Res.* **29**:292-298.

Doering, L.C. (1994): Nervous system modification by transplants and gene transfer. *BioEssays* **16**:825-831.

Dom, R., Brucher, J.M., Ceuterick, C., Carton, H. and Martin, J.J. (1979): Adult ceroid-lipofuscinosis (Kufs' disease) in two brothers. Retinal and visceral storage in one; Diagnostic muscle biopsy in the other. *Acta Neuropathol.* **45**:67-72.

Dowson, J.H., Armstrong, D., Koppang, N., Lake, B.D. and Jolly, R.D. (1982): Autofluorescence emission spectra of neuronal lipopigment in animal and human ceroidoses (ceroid-lipofuscinoses). *Acta Neuropathol.* **58**:152-156.

DuCerf, C., Bancel, B., Caillon, P., Adham, M., Guibaud, P., Spay, G. and Pouyet, M. (1992): Orthotopic liver transplantation for type I Gaucher's disease. *Transplantation* **53**:1141-1143.

Duncan, B.W., Harrison, M.R., Crombleholme, T.M., Clemons, G., Tavassoli, M. and Zanjani, E.D. (1992): Effect of erythropoietic stress on donor hematopoietic cell expression in chimeric rhesus monkeys transplanted in utero. *Exp. Hematol.* **20**:350-353.

Dunn, W.A., Raizada, M.K., Vogt, E.S. and Brown, E.A. (1994): Growth factor-induced neurite growth in primary neuronal cultures of dogs with neuronal ceroid lipofuscinosis. *Int. J. Devel. Neurosci.* **12**:185-196.

Dyer, M.R., Gay, N.J. and Walker, J.E. (1989): DNA sequences of a bovine gene and of two related pseudogenes for the proteolipid subunit of mitochondrial ATP synthase. *Biochem. J.* **260**:249-258.

Dyer, M.R. and Walker, J.E. (1993): Sequences of members of the human gene family for the c subunit of mitochondrial ATP synthase. *Biochem. J.* **293**:51-64.

Dyken, P.R. and Krawiecke, N. (1983): Neurodegenerative disease of infancy and childhood. *Ann. Neurol.* **13**:351-364.

Dyken, P.R. (1988): Reconsideration of the classification of the neuronal ceroid-lipofuscinoses. *Am. J. Med. Genet., Suppl.* **5**:69-84.

Dyken, P.R. (1989): The neuronal ceroid lipofuscinoses. *J. Child Neurol.* **4**:165-174.

Edathodu, A.K., Dyken, P.R., Trefz, J.I. and Kelloes, C.L. (1984): Two new forms of neuronal ceroid-lipofuscinosis: Expanded clinical, morphologic and biochemical classification. *Neurology* **34**:150.

Edwards, J.F., Storts, R.W., Joyce, J.R., Shelton, J.M. and Menzies, C.S. (1994): Juvenile-onset neuronal ceroid-lipofuscinosis in Rambouillet sheep. *Vet. Pathol.* **31**:48-54.

Eiberg, H., Gardiner, R.M. and Mohr, J. (1989): Batten disease (Spielmeyer-Sjøgren disease) and haptoglobins (HP): indication of linkage and assignment to chr. 16. *Clin. Genet.* **36**:217-218.

Einerhand, M.P.W. and Valerio, D. (1992): Gene transfer into hematopoietic stem cells: Prospects for human gene therapy. *Curr. Top. Microbiol. Immunol.* **177**:217-235.

Ekhterae, D., Crumbleholme, T., Karson, E., Harrison, M.R., Anderson, W.F. and Zanjani, E.D. (1990): Retroviral vector-mediated transfer of the bacterial neomycin resistance gene into fetal and adult sheep and human hematopoietic progenitors in vitro. *Blood* **75**:365-369.

Eldred, G.E., Miller, G.V., Stark, W.S. and Feeney-Burns, L. (1982): Lipofuscin: Resolution of discrepant fluorescence data. *Science* **216**:757-759.

Erbe, R.W. (1974): Therapy in genetic disease. *New Engl. J. Med.* **291**:1028-1029.

Ezaki, J., Wolfe, L.S. and Kominami, E. (1993): Molecular basis of lysosomal accumulation of subunit c of mitochondrial ATP synthase in neuronal ceroid-lipofuscinosis. *J. Inher. Metab. Dis.* **16**:296-298.

Ezaki, J., Wolfe, L.S., Higuti, T., Ishidoh, K. and Kominami, E. (1995): Specific delay of degradation of mitochondrial ATP synthase subunit c in late infantile neuronal ceroid-lipofuscinosis (Batten disease). *Am. J. Med. Genet.* **56**:(In press).

Fankhauser, R. (1965): Degenerative lipidiotische Erkrankung des Zentralnervensystems bei zwei Hunden. *Schweiz. Arch. Tierheilk.* **107**:73 Cited by Riis, R.C., Cummings, J.F., Loew, E.R., de Lahunta, A. (1992): Tibetan terrier model of canine ceroid-lipofuscinosis. *Am. J. Med. Genet.* **42**:615-621.

Faust, J.R., Rodman, J.S., Daniel, P.F., Dice, J.F. and Bronson, R.T. (1994): Two related proteolipids and dolichol-linked oligosaccharides accumulate in motor neuron degeneration mice *(mnd/mnd)*, a model for neuronal ceroid lipofuscinosis. *J. Biol. Chem.* **269**:10150-10155.

Fearnley, I.M., Walker, J.E., Martinus, R.D., Jolly, R.D., Kirkland, K.B., Shaw, G.J. and Palmer, D.N. (1990): The sequence of the major protein stored in ovine ceroid lipofuscinosis is identical with that of the dicyclohexylcarbodiimide-reactive proteolipid of mitochondrial ATP synthase. *Biochem. J.* **268**:751-758.

Fillingame, R.H. (1981): Biochemistry and genetics of bacterial H^+-translocating ATPases. *Curr. Top. Bioenerg.* **11**:35-106.

Fiske, R. A. and Storts, R.W. (1988): Neuronal ceroid-lipofuscinosis in Nubian goats. *Vet. Pathol.* **25**:171-173.

Flake, A.W., Harrison, M.R., Adzick, N.S. and Zanjani, E.D. (1986): Transplantation of fetal hematopoietic stem cells *in utero:* The creation of hematopoietic chimeras. *Science* **233**:776-778.

Folch, J. and Lees, M. (1951): Proteolipides: A new type of tissue lipoproteins. Their isolation from brain. *J. Biol. Chem.* **191**:807-817.

Foster, D.L. and Fillingame, R.H. (1982): Stoichiometry of subunits in the H^+-ATPase complex of *Escherichia coli*. *J. Biol. Chem.* **257**:2009-2015.

Fratantoni, J.C., Hall, C.W. and Neufeld, E.F. (1968): Hurler and Hunter syndromes: Mutual correction of the defect in cultured fibroblasts. *Science* **162**:570-572.

Garborg, I., Torvik, A., Hals, J., Tangsrud, S.E. and Lindemann, R. (1987): Congenital neuronal ceroid lipofuscinosis. *Acta Pathol. Microbiol. Immunol. Scand.* **95**:119-125.

Gardiner, R.M. (1992): Mapping the gene for juvenile onset neuronal ceroid lipofuscinosis to chromosome 16 by linkage analysis. *Am. J. Med. Genet.* **42**:539-541.

Garrod, A.E. (1908). In-born errors of metabolism. (Croonian lectures) *Lancet* **2**: 1, 73, 142, 214. Cited by Beaudet, A.L., Scriver, C.R., Sly, W.S., Valle, D., Cooper, D.N., Mc Kusick, V.A. and Schmidke, J. (1989): Genetics and biochemistry of variant human phenotypes. In *The Metabolic Basis of Inherited Disease.* Sixth edition. (Eds. Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D.). pp. 3-53. McGraw-Hill Inc., New York.

Gasper, P.W., Thrall, M.A., Wenger, D.A., Macy, D.W., Ham, L., Dornsife, R.E., McBiles, K., Quackenbush, S.L., Kesel, M.L., Gillette E.L. and Hoover, E.A. (1984): Correction of feline arylsulphatase B deficiency (mucopolysaccharidosis VI) by bone marrow transplantation. *Nature* **312**:467-469.

Gatti, R.A., Meeuwissen, H.J., Allen, H.D., Hong, R. and Good, R.A. (1968): Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet* **ii**:1366-1369.

Gautheron, D.C. and Godinot, C. (1988): Evidence from immunological studies of structure-mechanism relationship of F_1 and F_1F_0. *J. Bioenerg. Biomembr.* **20**:451-468.

Gay, N.J. and Walker, J.E. (1985): Two genes encoding the bovine mitochondrial ATP synthase proteolipid specify precursors with different import sequences and are expressed in a tissue-specific manner. *EMBO J.* **4**:3519-3524.

Gillis, W. S., Bennett, M. J., Galloway, J. H., Cartwright, I. J., Hosking, G. P. and Smith, C. M. L. (1987): Lipid abnormalities in Batten's disease. *J. Inher. Metab. Dis.* **10**:329-332.

Godel, V., Blumenthal, M., Goldman, B., Keren, G. and Padeh, B. (1978): Visual functions in Tay Sachs diseased patients following enzyme replacement therapy. *Metabol. Ophthalmol.* **2**:27-32.

Godinot, C. and Di Pietro, A. (1986): Structure and function of the ATPase-ATP synthase complex of mitochondria as compared to chloroplasts and bacteria. *Biochimie* **68**:367-374.

Goebel, H.H. and Dahme, E. (1985): Retinal ultrastructure of neuronal ceroid-lipofuscinosis in the Dalmatian dog. *Acta Neuropathol.* **68**:224-229.

Goebel, H.H. and Dahme, E. (1986): Ultrastructure of retinal pigment epithelial and neural cells in the neuronal ceroid-lipofuscinosis affected Dalmatian dog. *Retina* **6**:179-187.

Goebel, H.H., Zeman, W., Patel, V.K., Pullarkat, R.K. and Lenard, H.G. (1979): On the ultrastructural diversity and essence of residual bodies in neuronal ceroid-lipofuscinosis. *Mech. Ageing Dev.* **10**:53-70.

Goebel, H.H., Bilzer, T., Dahme, E. and Malkusch, F. (1988): Morphological studies in canine (Dalmatian) neuronal ceroid-lipofuscinosis. *Am. J. Med. Genet., Suppl.* **5**:127-139.

Goebel, H.H. (1993): Protracted juvenile neuronal ceroid-lipofuscinosis. *J. Inher. Metab. Dis.* **16**:233-236.

Gonatas, N.K., Terry, R.D., Winkler, R., Korey, S.R., Gomez, C.J. and Stein, A. (1963): A case of juvenile lipidosis: The significance of electron microscopic and biochemical observations of a cerebral biopsy. *J. Neuropathol. Exp. Neurol.* **22**:557-580. Cited by Zeman, W. (1970): Historical development of the nosological concept of amaurotic familial idiocy. In *Handbook of Clinical Neurology,* (Eds. Vinken, P.J. and Bruyn, G.W.). pp. 212-232. North Holland Publishing Co., Amsterdam.

Graydon, R.J. and Jolly, R.D. (1984): Ceroid-lipofuscinosis (Batten's disease). Sequential electrophysiologic and pathologic changes in the retina of the ovine model. *Invest. Ophthalmol. Vis. Sci.* **25**:294-301.

Green, P.D. and Little, P.B. (1974): Neuronal ceroid-lipofuscin storage in Siamese cats. *Can. J. Comp. Med.* **38**:207-212.

Greene, H.L., Hug, G. and Schubert, W.K. (1969): Metachromatic Leukodystrophy. Treatment with arylsulfatase-A. *Arch. Neurol.* **20**:147-153.

Gregoriadis, G., Neerunjun, D., Meade, T.W., Goolamali, S.K., Weereratne, H. and Bull, G. (1980): Experiences after long-term treatment of a type I Gaucher disease patient with liposome-entrapped glucocerebroside: β-glucosidase. *Birth Defects* **16**:383-392.

Grisolia, S., Hernandez-Yago, J. and Knecht, E. (1985): Regulation of mitochondrial protein concentration: A plausible model which may permit assessing protein turnover. *Curr. Top. Cell. Regul.* **27**:387-396.

Groth, C.G., Hagenfeldt, L., Dreborg, S., Löfström, B., Öckerman, P.A., Samuelsson, K., Svennerholm, L., Werner, B. and Westberg, G. (1971): Splenic transplantation in a case of Gaucher's disease. *Lancet* **i**:1260-1264.

Groth, C.-G., Collste, H., Dreborg, S., Håkansson, G., Lundgren, G. and Svennerholm, L. (1979): Attempt at enzyme replacement by organ transplantation: Renal transplantation in Gaucher disease. *Transplant. Proc.* **11**:1218-1219.

Guilbault, G.G. (1990): General aspects of luminescence spectroscopy. In *Practical Fluorescence.* (Ed. Guilbault, G.G.). Second edition. pp. 1-40. Marcel Dekker Inc., New York.

Gültekin, H. and Heermann, K.H. (1988): The use of polyvinylidenedifluoride membranes as a general blotting matrix. *Anal. Biochem.* **172**:320-329.

Gutteridge, J.M.C., Rowley, D.A., Halliwell, B. and Westermarck, T. (1982a): Increased non-protein-bound iron and decreased protection against superoxide-radical damage in cerebrospinal fluid from patients with neuronal ceroid lipofuscinoses. *Lancet* **ii**:459-460.

Gutteridge, J.M.C., Kerry, P.J. and Armstrong, D. (1982b): Autoxidized and lipoxidase-treated polyunsaturated fatty acids autofluorescence associated with the decomposition of lipid peroxides. *Biochim. Biophys. Acta* **711**:460-465.

Hagberg, B., Hultquist, G., Öhman, R. and Svennerholm, L. (1965): Congenital amaurotic idiocy. *Acta Paediatr. Scand.* **54**:116-130.

Hall, N.A. and Patrick, A.D. (1985): Dolichol and phosphorylated dolichol content of tissues in ceroid-lipofuscinosis. *J. Inher. Metab. Dis.* **8**:178-183.

Hall, N.A. and Patrick, A.D. (1988): Accumulation of dolichol-linked oligosaccharides in ceroid-lipofuscinosis (Batten disease). *Am. J. Med. Genet., Suppl.* **5**:221-232.

Hall, N.A., Jolly, R.D., Palmer, D.N., Lake, B.D. and Patrick, A.D. (1989): Analysis of dolichyl pyrophosphoryl oligosaccharides in purified storage cytosomes from ovine ceroid-lipofuscinosis. *Biochim. Biophys. Acta* **993**:245-251

Hall, N.A., Lake, B.D., Palmer, D.N., Jolly, R.D. and Patrick, A.D. (1990): Glycoconjugates in storage cytosomes from ceroid-lipofuscinosis (Batten's disease) and in lipofuscin from old-age brain. In *Lipofuscin and Ceroid Pigments.* (Ed. Porta, E.A.). pp. 225-242. Plenum Press, New York.

Hall, N.A., Lake, B.D. and Patrick, A.D. (1991a): Recent biochemical and genetic advances in our understanding of Batten's disease (ceroid-lipofuscinosis). *Dev. Neurosci.* **13**:339-344.

Hall, N.A., Lake, B.D., Dewji, N.N. and Patrick, A.D. (1991b): Lysosomal storage of subunit c of mitochondrial ATP synthase in Batten disease (ceroid-lipofuscinosis). *Biochem. J.* **275**:269-272.

Hall, N.A., Thomas-Oates, J.E., Dell, A., Haltia, M., Lake, B.D. and Patrick, A.D. (1992): Stored dolichyl pyrophosphoryl oligosaccharides in Batten disease. *Am. J. Med. Genet.* **42**:580-585.

Haltia, M., Rapola, J. and Santavuori, P. (1973): Infantile type of so-called neuronal ceroid-lipofuscinosis. Histological and electron microscopical studies. *Acta Neuropathol.* **26**:157-170.

Hänichen, T. and Püschner, H. (1971): Generalised ceroid-lipofuscinosis with neural complications in a dog. *Vet. Med. Rev.* **1**:27-39.

Harden, A. and Pampiglione, G. (1982): Neurophysiological studies (EEG/ERG/VEP/ SEP) in 88 children with so-called neuronal ceroid-lipofuscinosis. In *Ceroid-lipofuscinosis (Batten's disease).* (Eds. Armstrong, D., Koppang, N., and Rider, J.A.). pp. 61-70. Elsevier Biomedical Press, Amsterdam.

Hare, J.F. (1990): Mechanisms of membrane protein turnover. *Biochim. Biophys. Acta* **1031**:71-90.

Harper, P.A.W., Walker, K.H., Healy, P.J., Hartley, W.J., Gibson, A.J. and Smith, J.S. (1988): Neurovisceral ceroid-lipofuscinosis in blind Devon cattle. *Acta Neuropathol.* **75**:632-636.

Harrison, M.R., Slotnick, R.N., Crombleholme, T.M., Golbus, M.S., Tarantal, A.F. and Zanjani, E.D. (1989): In-utero transplantation of fetal liver haemopoietic stem cells in monkeys. *Lancet* **ii**:1425-1427.

Hartl, F.-U., Pfanner, N., Nicholson, D. W. and Neupert, W. (1989): Mitochondrial protein import. *Biochim. Biophys. Acta* **988**:1-45.

Hatefi, Y. (1985): The mitochondrial electron transport and oxidative phosphorylation system. *Ann. Rev. Biochem.* **54**:1015-1069.

Hendrick, J.P., Hodges, P.E. and Rosenberg, L.E. (1989): Survey of amino-terminal proteolytic cleavage sites in mitochondrial precursor proteins: Leader peptides cleaved by two matrix proteases share a three-amino acid motif. *Proc. Natl. Acad. Sci. USA* **86**:4056-4060.

Hickey, W.F. and Kimura, H. (1988): Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. *Science* **239**:290-292.

Hickey, W.F., Vass, K. and Lassmann, H. (1992): Bone marrow-derived elements in the central nervous system: An immunohistochemical and ultrastructural survey of rat chimeras. *J. Neuropathol. Exp. Neurol.* **51**:246-256.

Hobbs, J.R. (1981): Bone marrow transplantation for inborn errors. *Lancet* **ii**:735-739.

Hockenbery, D., Nuñez, G., Milliman, C., Schreiber, R.D. and Korsmeyer, S.J. (1990): Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* **348**:334-336.

Hoogerbrugge, P.M., Suzuki, K., Suzuki, K., Poorthuis, B.J.H.M., Kobayashi, T., Wagemaker, G. and van Bekkum, D.W. (1988): Donor-derived cells in the central nervous system of twitcher mice after bone marrow transplantation. *Science* **239**:1035-1038.

Hoover, D.M., Little, P.B. and Cole, W.D. (1984): Neuronal ceroid-lipofuscinosis in a mature dog. *Vet. Pathol.* **21**:359-361.

Holländer, G.A., Luskey, B.D., Williams, D.A. and Burakoff, S.J. (1992): Functional expression of human CD8 in fully reconstituted mice after retroviral-mediated gene transfer of hemopoietic stem cells. *J. Immunol.* **149**:438-444.

Howell, J.McC., Dorling, P.R., DiMarco, P.N. and Taylor, E.G. (1987): Multiple implantation of normal amnion into cattle with generalized glycogenosis type II. *J. Inher. Metab. Dis.* **10**:3-10.

Howell, J.McC., Dorling, P.R., Di Marco, P.N., Palmer, D., Taylor, E.G. and Shelton, J.N. (1990): Some experiences of enzyme replacement therapy in generalised glycogenosis type II of bovines. In *Pathogenesis and Therapy of Duchenne and Becker Muscular Dystrophy*. (Eds. Kakulas, B.A. and Mastaglia, F.L.). pp. 119-128. Raven Press Ltd., New York.

Hudson, L.D.S., Fiddler, M.B. and Desnick, R.J. (1980): Immunologic aspects of enzyme replacement therapy. An evaluation of the immune response to unentrapped, erythrocyte- and liposome-entrapped enzyme in C3H/HeJ Gus mice. *Birth Defects* **16:**163-178.

Humpreys, S., Lake, B.D. and Schultz, C.L. (1985): Congenital amaurotic idiocy- A pathological, histochemical, biochemical and ultrastructural study. *Neuropathol. Appl. Neurobiol.* **11**:475-484.

Issartel, J.P., Dupuis, A., Garin, J., Lunardi, J., Michel, L. and Vignais, P.V. (1992): The ATP synthase (F_o-F_1) complex in oxidative phosphorylation. *Experientia* **48**:351-362.

Jansky, J. (1908): Dosud neposany pripad familiarni amauroticke idiotie komplikovane s hypoplasii mozeckovou. *Sb. Lek.* **13**:165-196. Cited by Dyken, P.R. (1988): Reconsideration of the classification of the neuronal ceroid-lipofuscinoses. *Am. J. Med. Genet., Suppl.* **5**:69-84.

Järplid, B. and Haltia, M. (1993): An animal model of the infantile type of neuronal ceroid-lipofuscinosis. *J. Inher. Metab. Dis.* **16**:274-277.

Järvelä, I., Vesa, J., Santavuori, P., Hellsten, E. and Peltonen, L. (1992): Molecular genetics of the neuronal ceroid-lipofuscinoses. *Pediatr. Res.* **32**:645-648.

Jolly, R.D., Thompson, K.G., Murphy, C.E., Manktelow, B.W., Bruere, A.N. and Winchester, B.G. (1976): Enzyme replacement therapy- An experiment of nature in a chimeric mannosidosis calf. *Pediatr. Res.* **10**:219-224.

Jolly, R.D. and West, D.M. (1976): Blindness in South-Hampshire sheep: A neuronal ceroid-lipofuscinosis. *NZ Vet. J.* **24**:123.

Jolly, R.D. and Hartley, W.J. (1977): Storage diseases of domestic animals. *Aust. Vet. J.* **53**:1-8.

Jolly, R.D., Janmaat, A., West, D.M. and Morrison, I. (1980): Ovine ceroid-lipofuscinosis: A model of Batten's disease. *Neuropathol. Appl. Neurobiol.* **6**:195-209.

Jolly, R.D., Janmaat, A., Graydon, R.J. and Clemett, R.S. (1982): Ceroid-lipofuscinosis: The ovine model. In *Ceroid-lipofuscinosis (Batten's disease).* (Eds. Armstrong, D., Koppang, N. and Rider, J.A.) pp.219-228. Elsevier Biomedical Press, Amsterdam, New York, Oxford.

Jolly, R.D., Shimada, A., Craig, A.S., Kirkland, K.B. and Palmer, D.N. (1988): Ovine ceroid-lipofuscinosis II: Pathologic changes interpreted in light of biochemical observations. *Am. J. Med Genet., Suppl.* **5**:159-170.

Jolly, R.D., Shimada, A., Dopfmer, I., Slack, P.M., Birtles, M.J. and Palmer, D.N. (1989): Ceroid-lipofuscinosis (Batten's disease): Pathogenesis and sequential neuropathological changes in the ovine model. *Neuropathol. Appl. Neurobiol.* **15**:371-383.

Jolly, R.D., Martinus, R.D., Shimada, A., Fearnley, I.M. and Palmer, D.N. (1990): Ovine ceroid-lipofuscinosis is a proteolipid proteinosis. *Can. J. Vet. Res.* **54**:15-21.

Jolly, R.D., Martinus, R.D. and Palmer, D.N. (1992): Sheep and other animals with ceroid-lipofuscinoses: Their relevance to Batten disease. *Am. J. Med. Genet.* **42**:609-614.

Jolly, R.D., Palmer, D.N., Studdert, V.P., Sutton, R.H., Kelly, W.R., Koppang, N., Dahme, G., Hartley, W.J., Patterson, J.S. and Riis, R.C. (1994): Canine ceroid-lipofuscinoses: A review and classification. *J. Small Anim. Pract.* **35**:299-306.

Jolly, R.D. (1995): Comparative biology of the neuronal ceroid-lipofuscinoses (NCL): An overview. *Am. J. Med. Genet.* **56**:(In press).

Jolly, R.D. and Palmer, D.N. (1995): The neuronal ceroid-lipofuscinoses (Batten disease): Comparative aspects. *Neuropathol. Appl. Neurobiol.* **21**:50-60.

Joshi, U.M., Kodavanti, P.R.S., Lockard, V.G. and Mehendale, H.M. (1989): Fluorescence studies on binding of amphiphilic drugs to isolated lamellar bodies: Relevance to phospholipidosis. *Biochim. Biophys. Acta* **1004**:309-320.

Kane, J.F. and Hartley, D.L. (1988): Formation of recombinant protein inclusion bodies in *Escherichia coli. TIBTEC* **6**:95-101.

Kantoff, P.W., Flake, A.W., Eglitis, M.A., Scharf, S., Bond, S., Gilboa, E., Erlich, H., Harrison, M.R., Zanjani, E.D. and Anderson, W.F. (1989): In utero gene transfer and expression : A sheep transplantation model. *Blood* **73**:1066-1073.

Katz, M.L., Eldred, G.E., Siakotos, A.N. and Koppang, N. (1988): Characterization of disease-specific brain fluorophores in ceroid-lipofuscinosis. *Am. J. Med. Genet., Suppl.* **5**:253-264.

Katz, M.L. and Gerhardt, K.O. (1990): Storage protein in hereditary ceroid-lipofuscinosis contains *s*-methylated methionine. *Mech. Ageing Dev.* **53**:277-290.

Katz, M.L. and Rodrigues, M. (1991): Juvenile ceroid-lipofuscinosis. Evidence for methylated lysine in neural storage body protein. *Am. J. Pathol.* **138**:323-332.

Katz, M.L. and Gerhardt, K.O. (1992): Methylated lysine in storage body protein of sheep with hereditary ceroid-lipofuscinosis. *Biochim. Biophys. Acta* **1138**:97-108.

Kida, E., Wisniewski, K.E. and Golabek, A.A. (1993): Increased expression of subunit c of mitochondrial ATP synthase in brain tissue from neuronal ceroid lipofuscinoses and mucopolyasccharidosis cases but not in long-term fibroblast cultures. *Neurosci. Lett.* **164**:121-124.

Kikugawa, K., Beppu, M., Kato, T., Yamaki, S. and Kasai, H. (1994): Accumulation of autofluorescent yellow lipofuscin in rat tissues estimated by sodium dodecylsulfate extraction. *Mech. Ageing Dev.* **74**:135-148.

Kitaguchi, T., Wisniewski, K.E., Maslinski, S., Maslinska, D., Wisniewski, T.M. and Kim, K.S. (1990): β-protein immunoreactivity in brains of patients with neuronal ceroid lipofuscinosis: ultrastructural and biochemical demonstration. *Neurosci. Lett.* **112**:155-160.

Knudson, A.G., Di Ferrante, N. and Curtis, J.E. (1971): Effect of leukocyte transfusion in a child with type II mucopolysaccharidosis. *Proc. Natl. Acad. Sci. USA* **68**:1738-1741.

Kohli-Kumar, M., Shahidi, N.T., Broxmeyer, H.E., Masterson, M., Delaat, C., Sambrano, J., Morris, C., Auerbach, A.D. and Harris, R.E. (1993): Haemopoietic stem/progenitor cell transplant in Fanconi anaemia using HLA-matched sibling umbilical cord blood cells. *Brit. J. Haematol.* **85**:419-422.

Kohlschütter, A., Hübner, C. and Gärtner, J. (1988): Decreased membrane fluidity of lymphocytes from patients with juvenile neuronal ceroid-lipofuscinosis. *Am. J. Med. Genet., Suppl.* **5**:203-207.

Kohlschütter, A., Gardiner, R.M. and Goebel, H.H. (1993): Human forms of neuronal ceroid-lipofuscinosis (Batten disease): Consensus on diagnostic criteria, Hamburg 1992. *J. Inher. Metab. Dis.* **16**:241-244.

Kominami, E., Ezaki, J., Muno, D., Ishido, K., Ueno, T. and Wolfe, L.S. (1992): Specific storage of subunit *c* of mitochondrial ATP synthase in lysosomes of neuronal ceroid lipofuscinosis (Batten's Disease). *J. Biochem.* **111**:278-282.

Koppang, N. (1970): Neuronal ceroid-lipofuscinosis in English setters. Juvenile amaurotic familiar idiocy (AFI) in English setters. *J. Small Anim. Pract.* **10**:639-644.

Koppang, N. (1973/74): Canine ceroid-lipofuscinosis- A model for human neuronal ceroid-lipofuscinosis and aging. *Mech. Ageing Dev.* **2**:421-445.

Koppang, N. (1987): Hereditary ceroid lipofuscinosis in English setter dogs. A model for human neuronal ceroid-lipofuscinosis and aging. In *Advances in Age Pigments Research,* Volume 64. (Eds. Totaro, E.A., Glees, P. and Pisanti, F.A.). pp. 185-195. Pergamon Press, Oxford.

Koppang, N. (1988): The English Setter with ceroid-lipofuscinosis: A suitable model for the juvenile type of ceroid-lipofuscinosis in humans. *Am. J. Med. Genet., Suppl.* **5**:117-125.

Koppang, N. (1992): English setter model and juvenile ceroid-lipofuscinosis in man. *Am. J. Med. Genet.* **42**:599-604.

Koppang, N. (1993): The significance of animal models for human ceroid-lipofuscinosis. *J. Inher. Metab. Dis.* **16**:272-273.

Krivit, W. and Shapiro, E.G. (1991): Bone marrow transplantation for storage diseases. In *Treatment of Genetic Diseases* (Ed. Desnick, R.J.). pp. 203-221. Churchill Livingstone Inc., London.

Krivit, W. and Shapiro, E.G. (1994): Bone marrow transplantation for storage diseases. In *Bone Marrow Transplantation* (Ed. Forman, S.J., Blume, K.G. and Thomas, E.D.). pp. 883-893. Blackwell Scientific Publications, London.

Kyle, J.W., Birkenmeier, E.H., Gwynn, B., Vogler, C., Hoppe, P.C., Hoffmann, J.W. and Sly, W.S. (1990): Correction of murine mucopolysaccheridosis VII by a human β-glucuronidase transgene. *Proc. Natl. Acad. Sci. USA* **87**:3914-3918.

Laemmli, U.K. (1970): Clevage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685.

Lake, B.D. and Cavanagh, N.P.C. (1978): Early-juvenile Batten's disease - A recognisable sub-group distinct from other forms of Batten's disease. *J. Neurol. Sci.* **36**:265-271.

Lake, B.D. (1984): Lysosomal enzyme deficiencies. Batten's disease (neuronal ceroid-lipofuscinosis). In *Greenfields Neuropathology.* Fourth edition. (Eds. Hume Adams, J., Corsellis, J.A.N. and Duchen, L.W.). pp.491-572. Wiley-Medical, John Wiley and Sons, Inc., New York.

Lake, B.D. and Hall, N.A. (1993): Immunolocalization studies of subunit c in late-infantile and juvenile Batten disease. *J. Inher. Metab. Dis.* **16**:263-266.

Lake, B.D., Henderson, D.C., Oakhill, A. and Vellodi, A. (1995): Bone marrow transplantation in Batten's disease (neuronal ceroid-lipofuscinosis). Will it work? *Am. J. Med. Genet.* **56**:(In press).

Lane, S.C., Jolly, R.D. and Boustany, R.-M. (1995): Apoptosis associated with retinal degeneration in ovine neuronal ceroid-lipofuscinosis. *J. Cell. Biochem., Abstr. Suppl.* **19B(B8-429)**:314.

Lauterwein, J., Brown, L.R., Wüthrich, K. (1980): High-resolution ^{1}H-NMR studies of monomeric melittin in aqueous solution. *Biochim. Biophys. Acta* **622**:219-230.

Lazarow, P.B. and Moser, H.W. (1989): Disorders of peroxisome biogenesis. In *The Metabolic Basis of Inherited Disease.* Sixth edition. (Eds. Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D.). pp. 1479-1509. McGraw-Hill Inc., New York.

Lehn, P.M. (1990): Gene therapy using bone marrow transplantation: a 1990 update. *Bone Marrow Transplant.* **5**:287-293.

Lemischka, I.R., Raulet, D.H. and Mulligan, R.C. (1986): Developmental potential and dynamic behaviour of hematopoietic stem cells. *Cell* **45**:917-927.

Libert, J., Martin. J.-J. and Ceuterick, C. (1982): Protracted and atypical forms of ceroid-lipofuscinosis. In *Ceroid-lipofuscinosis (Batten's disease).* (Eds. Armstrong, D., Koppang, N. and Rider, J.A.). pp. 45-59. Elsevier Biomedical Press, Amsterdam.

Liley, A.W. (1963): Intrauterine transfusion of foetus in haemolytic disease. *Brit. Med. J.* **2**:1107-1109.

Lindvall, O., Brundin, P., Widner, H., Rehncrona, S., Gustavii, B., Frackowiak, R., Leenders, K.L., Sawle, G., Rothwell, J.C., Marsden, C.D. and Björklund, A. (1990): Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. *Science* **247**:574-577.

Lipsky, N.G. and Pedersen, P.L. (1981): Mitochondrial turnover in animal cells. Half lives of mitochondria and mitochondrial subfractions of rat liver based on [^{14}C]bicarbonate incorporation. *J. Biol. Chem.* **256**:8652-8657.

Lolley, R.N. (1994): The *rd* gene defect triggers programmed rod cell death. The Proctor Lecture. *Invest. Ophthalmol. Visual Sci.* **35**:4182-4191.

Loo, T.W. and Bragg, P.D. (1982): The DCCD-binding polypeptide is close to the F_1 ATPase-binding site on the cytoplasmic surface of the cell membrane of *Escherichia coli.* Biochem. *Biophys. Res. Comm.* **106**:400-406.

Luzikov, V.N. (1985): Mitochondrial degradation in the cell. In *Mitochondrial biogenesis and breakdown* (Ed. Luzikov, V. N.). pp.132-153. Consultants Bureau, New York.

Malkusch, F.L. (1984): Bioptic diagnosis of canine neuronal ceroid-lipofuscinosis (NCL). Doctoral Thesis Veterinary Medicine, University of Munich. Cited by: Jolly, R.D., Palmer, D.N., Studdert, V.P., Sutton, R.H., Kelly, W.R., Koppang, N., Dahme, G., Hartley, W.J., Patterson, J.S. and Riis, R.C. (1994): Canine ceroid-lipofuscinoses: A review and classification. *J. Small Anim. Pract.* **35**:299-306.

Mandel, M., Moriyama, Y., Hulmes, J. D., Pan, Y.-C. E., Nelson, H. and Nelson, N. (1988): cDNA sequence encoding the 16-kDa proteolipid of chromaffin granules implies gene duplication in the evolution of H^+-ATPases. *Proc. Natl. Acad. Sci. USA* **85**:5521-5524.

Manesis, E.K., Cameron, C.H. and Gregoriadis, G. (1979): Hepatitis B surface antigen-containing liposomes enhance humoral and cell-mediated immunity to the antigen. *FEBS Lett.* **102**:107-111.

Marsh, D., Watts, A., Maschke, W. and Knowles, P.F. (1978): Protein-immobilized lipid in dimyristoylphosphatidylcholine-substituted cytochrome oxidase: Evidence for both boundary and trapped-bilayer lipid. *Biochem. Biophys. Res. Comm.* **81**:397-402.

Martin, J.-J. (1993): Adult type of neuronal ceroid-lipofuscinosis. *J. Inher. Metab. Dis.* **16**:237-240.

Martinus, R.D. (1990): Biochemical studies on animal models of ceroid-lipofuscinoses. Doctoral Thesis in Veterinary Pathology, Massey University. p. 39.

Martinus, R.D., Harper, P.A.W., Jolly, R.D., Bayliss, S.L., Midwinter, G.G., Shaw, G.J. and Palmer, D.N. (1991): Bovine ceroid-lipofuscinosis (Batten's disease): The major component stored is the DCCD-reactive proteolipid, subunit *c*, of mitochondrial ATP synthase. *Vet. Res. Comm.* **15**:85-94.

Mayhew, I.G., Jolly, R.D., Pickett, B.T. and Slack, P.M. (1985): Ceroid-lipofuscinosis (Batten's disease): Pathogenesis of blindness in the ovine model. *Neuropathol. Appl. Neurobiol.* **11**:273-290.

Mayou, M.S. (1904): Cerebral degeneration with symmetrical changes in the macula in three members of a family. *Trans. Ophthalmol. Soc. US.* **24**:142-145. Cited by Dyken, P.R. (1988): Reconsideration of the classification of the neuronal ceroid-lipofuscinoses. *Am. J. Med. Genet., Suppl.* **5**:69-84.

Medd, S.M., Walker, J.E., Fearnley, I.M., Jolly, R.D. and Palmer, D.N. (1991): Lysosomal storage of a mitochondrial protein in Batten's disease (ceroid lipofuscinosis). In *Molecular basis of neurological disorders and their treatment.* (Eds. Garrod, J.W., Albano, D., Ferrari, E. and Papa, S.). pp. 154-165. Chapman and Hall, London.

Medd, S.M., Walker, J.E. and Jolly, R.D. (1993): Characterization of the expressed genes for subunit c of mitochondrial ATP synthase in sheep with ceroid lipofuscinosis. *Biochem. J.* **293**:65-73.

Messer, A., Plummer, J., MacMillen, M.C. and W.N. Frankel (1995): Genetics of primary and timing effects in the *mnd* mouse. *Am. J. Med. Genet.* **56**:(In press).

Migheli, A., Cavalla, P., Piva, R., Giordana, M.T. and Schiffer, D. (1994): bcl-2 protein expression in aged brain and neurodegenerative diseases. *NeuroReport* **5**: 1906-1908.

Mitchison, H.M., Taschner, P.E.M., O'Rawe, A.M., de Vos, N., Phillips, H.A., Thompson, A.D., Kozman, H.M., Haines, J.L., Schlumpf, K., D'Arigo, K., Boustany, R.-M.N., Callen, D.F., Breuning, M.H., Gardiner, R.M., Mole, S.E. and Lerner, T.J. (1994): Genetic mapping of the Batten disease locus *(CLN3)* to the interval *D16S288-D16S383* by analysis of haplotypes and allelic association. *Genomics* **22**:465-468.

Moen, R.C. (1991): Directions in gene therapy. *Blood Cells* **17**:407-416.

Mohrmann, R.L., Mah, V. and Vinters, H.V. (1990): Neuropathologic findings after bone marrow transplantation: An autopsy study. *Hum. Pathol.* **21**:630-639.

Mollay, C. and Kreil, G. (1973): Fluorometric measurements on the interaction of melittin with lecithin. *Biochim. Biophys. Acta* **316**:196-203.

Moroni-Rawson, P., Palmer, D.N., Jolly, R.D. and Jordan, T.W. (1995): Variant proteins in ovine ceroid-lipofuscinosis. *Am. J. Med. Genet.* **56**:(In press).

Moser, H.W., O'Brien, J.S., Atkins, L., Fuller, T.C., Kliman, A., Janowska, S., Russell, P.S., Bartsocas, C.S., Cosimi, B. and Dulaney, J.T. (1974): Infusion of normal HL-A identical leukocytes in Sanfilippo disease type B. Estimate of infused cell survival by assays of α-N-Acetyl-glucosaminidase activity and cytogenetic techniques: Effect on glycosaminoglycan excretion in the urine. *Arch. Neurol.* **31**:329-337.

Moullier, P., Bohl, D., Heard, J.-M. and Danos, O. (1993): Correction of lysosomal storage in the liver and spleen of MPS VII mice by implantation of genetically modified skin fibroblasts. *Nat. Genet.* **4**:154-159.

Muenzer, J., Neufeld, E.F., Constantopoulos, G., Caruso, R.F., Kaiser-Kupfer, M.I., Pikus, A., McDonald, H.D. and Zasloff, M.A. (1985): Amniotic membrane implantation in mucopolysaccharidosis. *Pediatr. Res.* **19**:251A.

Mulligan, R.C. (1993): The basic science of gene therapy. *Science* **260**:926-932.

Murnane, R.D., Wright, R.W., Ahern-Rindell, A.J. and Prieur, D.J. (1991): Prenatal lesions in an ovine fetus with GM_1 gangliosidosis. *Am. J. Med. Genet.* **39**:106-111.

Nakayama, H., Uchida, K., Shouda, T., Uetsuka, K., Sasaki, N. and Goto, N. (1993): Systemic ceroid-lipofuscinosis in a Japanese domestic cat. *J. Vet. Med. Sci.* **55**:829-831.

Neufeld, E.F. and Fratantoni, J.C. (1970): Inborn errors of mucopolysaccharide metabolism. Faulty degradative mechanisms are implicated in this group of human diseases. *Science* **169**:141-146.

Neufeld, E.F. and Cantz, M.J. (1971): Corrective factors for inborn errors of mucopolysaccharide metabolism. *Ann. NY Acad. Sci.* **179**:580-587.

Neufeld, E.F. (1991): Lysosomal storage diseases. *Ann. Rev. Biochem.* **60**:257-280.

Ng Ying Kin, N.M.K., Palo, J., Haltia, M. and Wolfe, L.S. (1983): High levels of brain dolichols in neuronal ceroid-lipofuscinosis and senescence. *J. Neurochem.* **40**:1465-1473.

Nimmo Wilkie, J.S. and Hudson, F.B. (1982): Neuronal and generalised ceroid-lipofuscinosis in a Cocker spaniel. *Vet. Pathol.* **19**:623-628.

Nishioka, J., Mizushima, T. and Ono, K. (1979): Treatment of mucopolysaccharidosis. Clinical and biochemical aspects of leucocyte transfusion as compared with plasma infusion in patients with Hurler's and Scheie's syndromes. *Clin. Orthopaed. Relat. Res.* **140**:194-203.

Nolta, J.A., Yu, X.J., Bahner, I. and Kohn, D.B. (1992): Retroviral-mediated transfer of the human glucocerebrosidase gene into cultured Gaucher bone marrow. *J. Clin. Invest.* **90**:342-348.

Norman, R.M. and Wood, N. (1941): Congenital form of amaurotic family idiocy. *J. Neurol. Psychiatr.* **4**:175-190. Cited by Dyken, P.R. (1988): Reconsideration of the classification of the neuronal ceroid-lipofuscinoses. *Am. J. Med. Genet., Suppl.* **5**:69-84.

Ohata, K., Marmarou, A. and Povlishock, J.T. (1990): An immunocytochemical study of protein clearance in brain infusion edema. *Acta Neuropathol.* **81**:162-177.

Palmer, D.N., Husbands, D.R. and Jolly, R.D. (1985): Phospholipid fatty acids in brains of normal sheep and sheep with ceroid-lipofuscinosis. *Biochim. Biophys. Acta* **834**:159-163.

Palmer, D.N., Husbands, D.R., Winter, P.J., Blunt, J.W. and Jolly, R.D. (1986a): Ceroid-lipofuscinosis in sheep I. Bis(monoacylglycero)-phosphate, dolichol, ubiquinone, phospholipids, fatty acids and fluorescence in liver lipopigment lipids. *J. Biol. Chem.* **261**:1766-1772.

Palmer, D.N., Barns, G., Husbands, D.R. and Jolly, R.D. (1986b): Ceroid-lipofuscinosis in sheep II. The major component of the lipopigment in liver, kidney, pancreas, and brain is low molecular weight protein. *J. Biol. Chem.* **261**:1773-1777.

Palmer, D.N., Martinus, R.D., Barns, G., Reeves, R.D. and Jolly, R.D. (1988). Ovine ceroid-lipofuscinosis I: Lipopigment composition is indicative of a lysosomal proteinosis. *Am. J. Med. Genet., Suppl.* **5**:141-158.

Palmer, D.N., Martinus, R.D., Cooper, S.M., Midwinter, G.G., Reid, J.C. and Jolly, R.D. (1989): Ovine ceroid lipofuscinosis. The major lipopigment protein and the lipid-binding subunit of mitochondrial ATP synthase have the same NH_2-terminal sequence. *J. Biol. Chem.* **264**:5736-5740.

Palmer, D.N., Fearnley, I.M., Medd, S.M., Walker, J.E., Martinus, R. D., Bayliss, S.L., Hall, N.A., Lake, B.D., Wolfe, L.S. and Jolly, R.D. (1990). Lysosomal storage of the DCCD reactive proteolipid subunit of mitochondrial ATP synthase in human and ovine ceroid lipofuscinoses. In *Lipofuscin and Ceroid Pigments* (Ed. Porta, E.A). pp. 211-223. Plenum Press, New York.

Palmer, D.N., Fearnley, I.M., Walker, J.E., Hall, N.A., Lake, B.D., Wolfe, L.S., Haltia, M., Martinus, R.D. and Jolly, R.D. (1992): Mitochondrial ATP synthase subunit *c* storage in the ceroid-lipofuscinoses (Batten disease). *Am. J. Med. Genet.* **42**:561-567.

Palmer, D.N., Bayliss, S.L., Clifton, P.A. and Grant, V.J. (1993): Storage bodies in the ceroid-lipofuscinoses (Batten disease): Low-molecular-weight components, unusual amino acids and reconstitution of fluorescent bodies from non-fluorescent components. *J. Inher. Metab. Dis.* **16**:292-295.

Palmer, D.N., Bayliss, S.L. and Westlake, V.J. (1995): Batten disease and the ATP synthase subunit c turnover pathway: Raising antibodies to subunit c. *Am. J. Med. Genet.* **56**: (In press).

Pampiglione, G. and Harden A. (1973): Neurophysiological identification of a late infantile form of 'neuronal lipidosis'. *J. Neurol. Neurosurg. Psychiat.* **36**:68-74.

Pardo, C.A., Rabin, B.A., Palmer, D.N. and Price, D.L. (1994): Accumulation of the adenosine triphosphate synthase subunit c in the *mnd* mutant mouse. *Am. J. Pathol.* **144**:829-235.

Parry, G., Palmer, D.N. and Williams, D.J. (1976): Ligand partitioning into membranes: Its significance in determining K_M and K_S values for cytochrome P-450 and other membrane bound receptors and enzymes. *FEBS Lett.* **67**:123-129.

Paton, B.C. and Poulos, A. (1984): Dolichol metabolism in cultured skin fibroblasts from patients with "neuronal" ceroid lipofuscinosis (Batten's disease). *J. Inher. Metab. Dis.* **7**:112-116.

Pearce, R.D., Kiehm, D., Armstrong, D.T., Little, P.B., Callahan, J.W., Klunder, L.R. and Clarke, J.T.R. (1989): Induction of hemopoietic chimerism in the caprine fetus by intraperitoneal injection of fetal liver cells. *Experientia* **45**:307-308.

Pearson, R.A. and Mellor, D.J. (1977): Changes in fetal fluid composition during the last 60 days of gestation in goats. *J. Reprod. Fertil.* **50**:171-173.

Perry, V.H., Hume, D.A. and Gordon, S. (1985): Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. *Neuroscience* **15**:313-326.

Pfaller, R., Steger, H.F., Rassow, J., Pfanner, N. and Neupert, W. (1988): Import pathways of precursor proteins into mitochondria: Multiple receptor sites are followed by a common membrane insertion site. *J. Cell Biol.* **107**:2483-2490.

Pfeifer, U. (1987): Functional morphology of the lysosomal apparatus. In *Lysosomes: Their role in protein breakdown* (Eds. Glaumann, H. and Ballard, F.J.). pp. 3-59. Academic Press, London.

Pickett, P., Dyer, K., Saunders, O. and Robertson, J. (1992): Ocular manifestation of ceroid-lipofuscinosis in a Spitz dog. *Vet. Pathol.* **29**:469.

Pullarkat, R.K. and Reha, H. (1982): Accumulation of dolichols in brains of elderly. *J. Biol. Chem.* **257**:5991-5993.

Pullarkat, R.K., Reha, H., Patel, V.K. and Goebel, H.H. (1982): Docosahexaenoic acid levels in brains of various forms of ceroid-lipofuscinosis. In *Ceroid-lipofuscinosis (Batten's disease).* (Eds. Armstrong, D., Koppang, N. and Rider, J.A.) pp.335-343. Elsevier Biomedical Press, Amsterdam, New York, Oxford.

Pullarkat, R.K., Kim, K.S., Sklower, S.L. and Patel, V.K. (1988): Oligosaccharyl diphosphodolichols in the ceroid-lipofuscinoses. *Am. J. Med. Genet., Suppl.* **5**:243-251.

Pullarkat, R.K. (1990): Biochemical basis of storage of oligosaccharyl diphosphodolichol in ceroid-lipofuscinoses. Abstract in: *Third International symposium on neuronal ceroid-lipofuscinoses.* Indianapolis, 1990.

Pullarkat, R.K. and Zawitosky, S.E. (1993): Glycoconjugate abnormalities in the ceroid-lipofuscinoses. *J. Inher. Metab. Dis.* **16**:317-322.

Purkiss, P., Gibbs, D.A. and Watts, R.W.E. (1983): Studies on the composition of urinary glycosaminoglycans and oligosaccharides in patients with mucopolysaccharidoses who were receiving fibroblast transplants. *Clin. Chim. Acta* **131**:109-121.

Rac, R. and Giesecke, P.R. (1975): Lysosomal storage disease in chihuahuas. *Aust. Vet. J.* **51**:403-404.

Rapola, J. (1993): Neuronal ceroid-lipofuscinoses in childhood. *Perspect. Pediatr. Pathol.* **17**:7-44.

Rattazzi, M.C., Lanse, S.B., McCullough, R.A., Nester, J.A. and Jacobs, E.A. (1980): Towards enzyme replacement in G_{M2} gangliosidosis: Organ disposition and induced central nervous system uptake of human β-hexosaminidase in the cat. *Birth Defects* **16**:179-193.

Read, W.K. and Bridges, C.H. (1969): Neuronal lipodystrophy. Occurrence in an inbred strain of cattle. *Vet. Pathol.* **6**:235-243.

Reddy, T.S., Birkle, D.L., Armstrong, D. and Bazan, N.G. (1985): Change in content, incorporation, and lipoxygenation of docosahexaenoic acid in retina and retinal pigment epithelium in canine ceroid-lipofuscinosis. *Neurosci. Lett.* **59**:67-72.

Rider, J.A. and Rider, D.L. (1988): Batten Disease: Past, Present, and Future. *Am. J. Med. Genet., Suppl.* **5**:21-26.

Riis, R.C. and Loew, E.R. (1988): Pathological notations of the retinal degenerations (rdi) in Tibetan terrier. *Proc. Am. Coll. Vet. Ophthalmol.* p. 88.

Riis, R.C., Cummings, J.F., Loew, E.R. and de Lahunta, A. (1992): Tibetan terrier model of canine ceroid-lipofuscinosis. *Am. J. Med. Genet.* **42**:615-621.

Sakuragawa, N., Yoshikawa, H. and Sasaki, M. (1992): Amniotic tissue transplantation: Clinical and biochemical evaluations for some lysosomal storage diseases. *Brain Dev.* **14**:7-11.

Sanadi, D.R. (1982): Mitochondrial coupling factor B. Properties and role in ATP synthesis. *Biochim. Biophys. Acta* **683**:39-56.

Sandbank, U. (1968): Congenital amaurotic idiocy. *Path. Europ.* **3**:226-229. Cited by Garborg, I., Torvik, A., Hals, J., Tangsrud, S.E. and Lindemann, R. (1987): Congenital neuronal ceroid lipofuscinosis. *Acta Pathol. Microbiol. Immunol. Scand.* **95**:119-125.

Sands, M.S., Barker, J.E., Vogler, C., Levy, B., Gwynn, B., Galvin, N., Sly, W.S. and Birkenmeier, E. (1993): Treatment of murine mucopolysaccharidosis type VII by syngeneic bone marrow transplantation in neonates. *Lab. Invest.* **68**:676-686.

Santavuori, P., Rapola, J., Sainio, K. and Raitta, Ch. (1982): A variant of Jansky-Bielschowsky disease. *Neuropediatrics* **13**:135-141.

Santavuori, P. (1988): Neuronal ceroid-lipofuscinoses in childhood. *Brain Dev.* **10**:80-83.

Santavuori, P., Rapola, J., Nuutila, A., Raininko, R., Lappi, M., Launes, J., Herva, R. and Sainio, K. (1991): The spectrum of Jansky-Bielschowsky disease. *Neuropediatrics* **22**:92-96.

Santavuori, P., Vanhanen, S.-L., Sainio, K., Nieminen, M., Wallden, T., Launes, J. and Raininko, R. (1993a): Infantile neuronal ceroid-lipofuscinosis (INCL): Diagnostic criteria. *J. Inher. Metab. Dis.* **16**:227-229.

Santavuori, P., Rapola, J., Raininko, R., Autti, T., Lappi, M., Nuutila, A., Launes, J. and Sainio, K. (1993b): Early juvenile neuronal ceroid-lipofuscinosis or variant Jansky-Bielschowsky disease: Diagnostic criteria and nomenclature. *J. Inher. Metab. Dis.* **16**:230-232.

Savukoski, M., Kestilä, M., Williams, R., Järvelä, I., Sharp, J., Santavuori, P., Gardiner, M. and Peltonen, L. (1995): The chromosomal assignment of CLN5 demonstrates that at least four genetic loci are involved in the pathogenesis of human ceroid lipofuscinoses. *Am. J. Med. Genet.* **56**:(In press).

Scaggiante, B., Comelli, M. and Romeo, D. (1991): Secretion of lysosomal hydrolases by cultured human amnion epithelial cells. *Exp. Cell Res.* **195**:194-198.

Schmidt, B., Wachter, E., Sebald, W. and Neupert, W. (1984): Processing peptidase of *Neurospora* mitochondria. Two-step cleavage of imported ATPase subunit 9. *Eur. J. Biochem.* **144**:581-588.

Schwartz, R. and Dameshek, W. (1959): Drug-induced immunological tolerance. *Nature* **183**:1682-1683.

Sebald, W., Hoppe, J. and Wachter, E. (1979): Amino acid sequence of the ATPase proteolipid from mitochondria, chloroplasts and bacteria (Wild type and mutants). In *Function and Molecular Aspects of Biomembrane Transport* (Eds. Quagliariello, E., Palmieri, F., Papa, S. and Klinkenberg, M.). pp. 63-74. Elsevier/North-Holland Biomedical Press, Amsterdam.

Sebald, W. and Hoppe, J. (1981): On the structure and genetics of the proteolipid subunit of the ATP synthase complex. *Curr. Top. Bioenerg.* **12**:1-64.

Senior, A.E. (1985): The proton-ATPase of *Escherichia coli*. *Curr. Top. Memb. Trans.* **23**:135-151.

Sharp, J., Savukoski, M., Wheeler, R., Järvelä, I., Peltonen, L., Gardiner, R.M. and Williams, R. (1995): Linkage analysis of late-infantile neuronal ceroid lipofuscinosis. *Am. J. Med. Genet.* **56**:(In press).

Shull, R.M., Hastings, N.E., Selcer, R.R., Jones, J.B., Smith, J.R., Cullen, W.C. and Constantopoulos, G. (1987): Bone marrow transplantation in canine mucopolysaccharidosis I: Effects within the central nervous system. *J. Clin. Invest.* **79**:435-443.

Shull, R.M., Breider, M.A. and Constantopoulos, G.C. (1988): Long-term neurological effects of bone marrow transplantation in a canine lysosomal storage disease. *Pediatr. Res.* **24**:347-352.

Siakotos, A.N., Goebel, H.H., Patel, V., Watanabe, I. and Zeman, W. (1972): The morphogenesis and biochemical characteristics of ceroid isolated from cases of neuronal ceroid-lipofuscinosis. In *Sphingolipids, Sphingolipodoses and Allied Disorders* (Eds. Volk, B.W. and Aronson, S.M.) pp.53-61. Plenum Press, New York.

Siakotos, A.N. and Koppang, N. (1973): Procedures for the isolation of lipopigments from brain, heart and liver, and their properties: A review. *Mech. Ageing Dev.* **2**:177-200.

Siakotos, A.N., Armstrong, D., Koppang, N. and Connole, E. (1978): Studies on the retina and the pigment epithelium in hereditary canine ceroid-lipofuscinosis. II. The subcellular distribution of lysosomal hydrolases and other enzymes. *Invest. Ophthalmol. Vis. Sci.* **17**:618-633.

Siakotos, A.N. and Munkres, K.D. (1981): Purification and properties of age pigments. In *Age Pigments,* (Ed. Sohal, R.S.) pp. 181-202. Elsevier North-Holland Biomedical Press, Amsterdam.

Siakotos, A.N., Bray, R., Dratz, E., van Kuijk, F., Sevanian, A. and Koppang, N. (1988): 4-Hydroxynonenal: A specific indicator for canine neuronal-retinal ceroidosis. *Am. J. Med. Genet., Suppl.* **5**:171-181.

Siakotos, A.N., van Kuijk, F.J.G.M. and Tischfield, J.A. (1993): Elevated levels of neutrophil 4-hydroxynonenal in canine neuronal ceroid-lipofuscinosis and human immortalized lymphocytes of NCL patients. *J. Inher. Metab. Dis.* **16**:323-329.

Sisk, D.B., Levesque, D.C., Wood, P.A. and Styer, E.L. (1990): Clinical and pathologic features of ceroid-lipofuscinosis in two Australian cattle dogs. *J. Am. Vet. Med. Assoc.* **197**:361-364.

Solomon, J.B. (1971): Foetal and Neonatal Immunology. In *North-Holland Research Monographs. Frontiers of Biology,* Volume 20. (Eds. Neuberger, A. and Tatum, E.L.). pp. 234-306. North-Holland Pub. Co., Amsterdam.

Spencer, D.D., Roabbins, R.J., Naftolin, F., Marek, K.L., Vollmer, T., Leranth, C., Roth, R.H., Price, L.H., Gjedde, A., Bunney, B.S., Sass, K.J., Elsworth, J.D., Kier, E.L., Makuch, R., Hoffer, P.B. and Redmond, D.E. (1992): Unilateral transplantation of human fetal mesencephalic tissue into the caudate nucleus of patients with Parkinson's disease. *New Engl. J. Med.* **327**:1541-1548.

Spielmeyer, W. (1905): Über familiäre amaurotische Idiotien. *Neurol. Cbl.* **24**:620-621. Cited by Dyken, P.R. (1988): Reconsideration of the classification of the neuronal ceroid-lipofuscinoses. *Am. J. Med. Genet., Suppl.* **5**:69-84.

Stengel, C. (1826): Eyr (Christiana) 1:347-352. Translated as an "Account of a singular illness among four siblings in the vicinity of Røraas". In *Ceroid-lipofuscinosis (Batten's disease).* (Eds. Armstrong, D., Koppang, N. and Rider, J.A.) pp.17-19. Elsevier Biomedical Press, Amsterdam.

Stevens, A. (1982): Pigments and minerals. In *Theory and Practice of Histological Techniques.* Second Edition. (Eds. Bancroft, J.D. and Stevens, A.) pp.242-266. Churchill Livingston Inc., London.

Studdert, V.P. and Mitten, R.W. (1991): Clinical features of ceroid-lipofuscinosis in Border collie dogs. *Aust. Vet. J.* **4**:137-140.

Suzuki, F., Johnson, A.B., Marquet, E. and Suzuki, K. (1968): A case of juvenile lipidosis: Electron microscopic, histochemical and biochemical studies. *Acta Neuropathol.* **11**:122-139.

Svennerholm, L., Hagberg, B., Haltia, M., Sournader, P. and Vanier, M.-T. (1975): Polyunsaturated fatty acid lipidosis. II. Lipid biochemical studies. *Acta Paediatr. Scand.* **64**:489-496.

Svennerholm, L. (1976): Polyunsaturated fatty acid lipidosis: A new nosological entity. In *Current Trends in Spingolipidoses and Allied Disorders.* (Eds. Volk, B.W. and Schneck, L.). pp. 389-402. Plenum Press, New York.

Tager, J.M., Hamers, M.N., Schram, A.W., Van den Bergh, F.A.J.T.M., Rietra, P.J.G.M., Loonen, C., Koster, J.F. and Slee, R. (1980): An appraisal of human trials in enzyme replacement therapy of genetic diseases. *Birth Defects* **16**:343-359.

Talbot, J.C., Dufourcq, J., de Bony J., Faucon, J.F., Lussan, C. (1979): Conformational change and self association of monomeric melittin. *FEBS Lett.* **102**:191-193.

Taylor, R.M., Farrow, B.R.H., Stewart, G.J. and Healy, P.J. (1986): Enzyme replacement in nervous tissue after allogeneic bone-marrow transplantation for fucosidosis in dogs. *Lancet* **ii**:772-774.

Taylor, R.M. and Farrow, B.R.H. (1988a): Ceroid-lipofuscinosis in Border collie dogs. *Acta Neuropathol.* **75**:627-631.

Taylor, R.M. and Farrow, B.R.H. (1988b): Animal model series. Fucosidosis. *Comp. Pathol. Bull.* **20**:2-5.

Taylor, R.M., Farrow, B.R.H., Stewart, G.J., Healy, P.J. and Tiver, K. (1988): The clinical effects of lysosomal enzyme replacement by bone marrow transplantation after total lymphoid irradiation on neurologic disease in fucosidase deficient dogs. *Transplant. Proc.* **20**:89-93.

Taylor, R.M. and Farrow, B.R.H. (1992): Ceroid-lipofuscinosis in the Border collie dog: Retinal lesions in an animal model of juvenile Batten disease. *Am. J. Med. Genet.* **42**:622-627.

Taylor, R.M., Farrow, B.R.H. and Stewart, G.J. (1992): Amelioration of clinical disease following bone marrow transplantation in fucosidase-deficient dogs. *Am. J. Med. Genet.* **42**:628-632.

Terasaki, P.I. (1991): Histocompatibility testing in transplantation. *Arch. Pathol. Lab. Med.* **115**:250-254.

Touraine, J.-L., Laplace, S., Rezzoug, F., Sanhadji, K., Veyron, P., Royo, C., Maire, I., Zabot, M.T., Vanier, M.T., Rolland, M.O., Goillot, E., de Bouteiller, O., Garnier, J.L., Pouteil-Noble, C., Raffaele, P., Livrozet, J.M. and Saint-Marc, T. (1991): The place of fetal liver transplantation in the treatment of inborn errors of metabolism. *J. Inher. Metab. Dis.* **14**:619-626.

Tylki-Szymanska, A., Maciejko, D., Kidawa, M., Jablonska-Budaj, U. and Czartoryska, B. (1985): Amniotic tissue transplantation as a trial of treatment in some lysosomal storage diseases. *J. Inher. Metab. Dis.* **8**:101-104.

Tyynelä, J. Palmer, D.N., Baumann, M. and Haltia, M. (1993): Storage of saposins A and D in infantile neuronal ceroid-lipofuscinosis. *FEBS Lett.* **330**:8-12.

Tzagoloff, A. and Meagher, P. (1971): Assembly of the mitochondrial membrane system. V. Properties of a dispersed preparation of the rutamycin-sensitive adenosine triphosphatase of yeast mitochondria. *J. Biol. Chem.* **246**:7328-7336.

Unger, E.R., Sung, J.H., Manivel, J.C., Chenggis, M.L., Blazar, B.R. and Krivit, W. (1993): Male donor-derived cells in the brains of female sex-mismatched bone marrow transplant recipients: A Y-chromosome specific *in situ* hybridization study. *J. Neuropathol. Exp. Neurol.* **52**:460-470.

Van Bagh, K., and Hortling, A.H. (1948): Blodfynovid juvenil amaurotisk idioti. *Nord. Med.* **38**:1072. Cited by Zeman, W. and Dyken, P. (1969): Neuronal ceroid-lipofuscinosis (Batten's disease): Relationship to amaurotic family idiocy? *Pediatrics* **44**:570-583.

Van den Bergh, F.A.J.T.M., Rietra, P.J.G.M., Kolk-Vegter, A.J., Bosch, E. and Tager, J.M. (1976): Therapeutic implications of renal transplantation in a patient with Fabry's disease. *Acta Med. Scand.* **200**:249-256.

Vandevelde, M. and Fatzer, R. (1980): Neuronal ceroid-lipofuscinosis in older Dachshunds. *Vet. Pathol.* **17**:686-692.

Vesa, J., Hellsten, E., Mäkelä, T.P., Strauss, J.F., Alitalo, K. and Peltonen, L. (1995): Towards the isolation of the CLN1 gene. *Am. J. Med. Genet.* **56**:(In press).

Vignais, P.V. and Satre, M. (1984): Recent developments on structural and functional aspects of the F_1 sector of H^+-linked ATPases. *Mol. Cell. Biochem.* **60**:33-70.

Vogt, H. (1905): Über familiäre amaurotische idiotie und verwandte Krankheitsbilder. *Monatsschr. Psychiatr. Neurol.* **18**:161-171, 310-357. Cited by Dyken, P.R. (1988): Reconsideration of the classification of the neuronal ceroid-lipofuscinoses. *Am. J. Med. Genet., Suppl.* **5**:69-84.

von Figura, K., Lögering, M. and Kresse, H. (1975): Serum α-N-acetylglucosaminidase: Determination, characterization and corrective activity in *Sanfilippi* B fibroblasts. *Z. Klin. Chem. Klin. Biochem.* **13**:285-289.

von Specht, B.U., Geiger, B., Arnon, R., Passwell, J., Keren, G., Goldman, B. and Padeh, B. (1979): Enzyme replacement in Tay-Sachs disease. *Neurology* **29**:848-854.

Walker, J.E., Fearnley, I.M. and Blows R.A. (1986): A rapid solid-phase protein microsequencer. *Biochem. J.* **237**:73-84.

Walkley, S.U., Thrall, M.A., Dobrenis, K., Huang, M., March, P.A., Siegel, D.A. and Wurzelmann, S. (1994): Bone marrow transplantation corrects the enzyme defect in neurons of the central nervous system in a lysosomal storage disease. *Proc. Natl. Acad. Sci. USA* **91**:2970-2974.

Walkley, S.U., March, P.A., Schroeder, C.E., Wurzelmann, S. and Jolly, R.D. (1995): Pathogenesis of brain dysfunction in Batten's disease. *Am. J. Med. Genet.* **56**:(In press).

Wallace, D.C. (1992): Mitochondrial Genetics: A paradigm for aging and degenerative diseases? *Science* **256**:628-632.

Walters, L.-R. (1986): The ethics of human gene therapy. *Nature* **320**:225-227.

Watabe, S. and Kimura, T. (1985): ATP-dependent protease in bovine adrenal cortex. Tissue specificity, subcellular localization, and partial characterization. *J. Biol. Chem.* **260**:5511-5517.

Wehry, E.L. (1990): Effects of molecular environment on fluorescence and phosphorescence. In *Practical Fluorescence.* Second edition. (Ed. Guilbault, G.G.). pp. 127-184. Marcel Dekker Inc., New York.

Westlake, V.J., Jolly, R.D., Bayliss, S.L. and Palmer, D.N. (1995): Immunocytochemical studies in the ceroid-lipofuscinoses (Batten disease) using antibodies to subunit c of mitochondrial ATP synthase. *Am. J. Med. Genet.* **56**:(In press).

Whitehead, B., Helms, P., Goodwin, M., Martin, I., Lask, B., Serrano, E., Scott, J.P., Smyth, R.L., Higenbottam, T.W., Wallwork, J., Elliot, M. and de Leval, M. (1991): Heart-lung transplantation for cystic fibrosis. 1: Assessment. *Arch. Dis. Childh.* **66**:1018-1021.

Wisniewski, K.E., Rapin, I. and Heaney-Kieras, J. (1988): Clinico-pathological variability in the childhood neuronal ceroid-lipofuscinoses and new observations on glycoprotein abnormalities. *Am. J. Med. Genet., Suppl.* **5**:27-46.

Wisniewski, K.E. and Maslinska, D. (1989): Immunoreactivity of ceroid lipofuscin storage pigment in Batten disease with monoclonal antibodies to the amyloid beta-protein. *New Engl. J. Med.* **320**:256-257.

Wisniewski, K.E., Kida, E., Gordon-Majszak, W. and Saitoh, T. (1990a): Altered amyloid β-protein precursor processing in brains of patients with neuronal ceroid lipofuscinosis. *Neurosci. Lett.* **120**:94-96.

Wisniewski, K.E., Maslinska, D., Kitaguchi, T., Kim, K.S., Goebel, H.H. and Haltia, M. (1990b): Topographic heterogeneity of amyloid β-protein epitopes in brains with various forms of neuronal ceroid lipofuscinoses suggesting defective processing of amyloid precursor protein. *Acta Neuropathol.* **80**:26-34.

Wisniewski, K.E. and Kida, E. (1990): Proteinase inhibitor alpha$_1$-anti-chymotrypsin has different expression in various forms of neuronal ceroid lipofuscinosis. *Exp. Neurol.* **110**:121-126.

Wisniewski, K.E., Gordon-Majszak, W., Maslinski, S. and Heaney-Kieras, J. (1992a): Altered protein patterns in brains of children with neuronal ceroid lipofuscinosis. *Am. J. Med. Genet.* **42**:568-574.

Wisniewski, K.E., Kida, E., Patxot, O.F. and Connell, F. (1992b): Variability in the clinical and pathological findings in the neuronal ceroid lipofuscinoses: Review of data and observations. *Am. J. Med. Genet.* **42**:525-532.

Wisniewski, K.E., Kida, E., Connell, F., Elleder, M., Eviatar, L. and Konkol, R.J. (1993): New subform of the late infantile form of neuronal ceroid lipofuscinosis. *Neuropediatrics* **24**:155-163.

Wisniewski, K.E., Golabek, A.A. and Kida, E. (1994): Increased urine concentration of subunit c of mitochondrial ATP synthase in neuronal ceroid lipofuscinoses patients. *J. Inher. Metab. Dis.* **17**:205-210.

Wolfe, L.S., Ng Ying Kin, N.M.K., Baker, R.R., Carpenter, S. and Andermann, F. (1977): Identification of retinoyl complexes as the autofluorescent component of the neuronal storage material in Batten disease. *Science* **195**:1360-1362.

Wolfe, L.S., Ng Ying Kin, N.M.K. and Baker, R.R. (1981): Batten disease and related disorders: New findings on the chemistry of the storage material. In *Lysosomes and Lysosomal Storage Diseases,* (Eds. Callahan, J.W. and Lowden, J.A.) pp.315-330. Raven Press, New York.

Wolfe, L.S. and Ng Ying Kin, N.M.K. (1982a): Batten disease: New research findings on the biochemical defect. *Birth Defects* **18**:233-239.

Wolfe, L.S., Ng Ying Kin, N.M.K., Palo, J. and Haltia, M. (1982b): Raised levels of cerebral cortex dolichols in Alzheimer's disease. *Lancet* **ii**:99.

Wolfe, L.S., Ng Ying Kin, N.M.K., Palo, J. and Haltia, M. (1983): Dolichols in brain and urinary sediment in neuronal ceroid lipofuscinosis. *Neurology* **33**:103-106.

Wolfe, L.S., Ivy, G.O. and Witkop, C.J. (1987): Dolichols, lysosomal membrane turnover and relationships to the accumulation of ceroid and lipofuscin in inherited diseases, Alzheimer's disease and aging. *Chemica Scripta* **27**:79-84.

Wolfe, L.S., Gauthier, S., Haltia, M. and Palo, J. (1988a): Dolichol and dolichyl phosphate in the neuronal ceroid-lipofuscinoses and other diseases. *Am. J. Med. Genet., Suppl.* **5**:233-242.

Wolfe, L.S., Gauthier, S. and Durham, H.D. (1988b): Dolichols and phosphorylated dolichols in the neuronal ceroid lipofuscinoses, other lysosomal storage diseases and Alzheimer disease. Induction of autolysosomes in fibroblasts. In *Lipofuscin-1987: State of the Art.* (Ed. Zs.-Nagy, I.). pp. 389-411. Elsevier Science Publishers, Amsterdam.

Wolfe, J.H., Schuchman, E.H., Stramm, L.E., Concaugh, E.A., Haskins, M.E., Aguirre, G.D., Patterson, D.F., Desnick, R.J. and Gilboa, E. (1990): Restoration of normal lysosomal function in mucopolysaccharidosis type VII cells by retroviral vector-mediated gene transfer. *Proc. Natl. Acad. Sci. USA* **87**:2877-2881.

Wolfe, J.H., Sands, M.S., Barker, J.E., Gwynn, B., Rowe, L.B., Vogler, C.A. and Birkenmeier, E.H. (1992a): Reversal of pathology in murine mucopolysaccharidosis type VII by somatic cell gene transfer. *Nature* **360**:749-753.

Wolfe, J.H., Deshmane, S.L. and Fraser, N.W. (1992b): Herpesvirus vector gene transfer and expression of β-glucuronidase in the central nervous system of MPS VII mice. *Nat. Genet.* **1**:379-384.

Wood, P.A., Sisk, D.B., Styer, E. and Baker, H.J. (1987): Animal model: Ceroidosis (ceroid-lipofuscinosis) in Australian cattle dogs. *Am. J. Med. Genet.* **26**:891-898.

Wu, D. and Keating, A. (1993): Hematopoietic stem cells engraft in untreated transplant recipients. *Exp. Hematol.* **21**:251-256.

Yan, W.L., Lerner, T.J., Haines, J.L., Gusella, J.F. (1995): Sequence analysis and mapping of a novel human mitochondrial ATP synthase subunit 9 cDNA. *Genomics*. (In press).

Yankner, B.A., Dawes, L.R., Fisher, S., Villa-Komaroff, L., Oster-Granite, M.L. and Neve, R.L. (1989): Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's disease. *Science* **245**:417-420.

Yeager, A.M., Brennan, S., Tiffany, C., Moser, H.W. and Santos, G.W. (1984): Prolonged survival and remyelination after hematopoietic cell transplantation in the twitcher mouse. *Science* **225**:1052-1054.

Yeager, A.M., Singer, H.S., Buck, J.R., Matalon, R., Brennan, S., O'Donnell O'Toole, S. and Moser, H.W. (1985): A therapeutic trial of amniotic epithelial cell implantation in patients with lysosomal storage diseases. *Am. J. Med. Genet.* **22**:347-355.

Yeager, A.M., Shinohara, M. and Shinn, C. (1991): Hematopoietic cell transplantation after administration of high-dose busulfan in murine globoid cell leukodystrophy (the twitcher mouse). *Pediatr. Res.* **29**:302-305.

Zanjani, E.D., Harrison, M.R. and Tavassoli, M. (1990): Enhancement of engraftment of in utero transplanted fetal liver hematopoietic stem cells (HSC) (abstract). *Blood* **76**:173.

Zanjani, E.D., Mackintosh, F.R. and Harrison, M.R. (1991): Hematopoietic chimerism in sheep and nonhuman primates by in utero transplantation of fetal hematopoietic stem cells. *Blood Cells* **17**:349-363.

Zanjani, E.D., Pallavicini, M.G., Ascensao, J.L., Flake, A.W., Langlois, R.G., Reitsma, M., MacKintosh, F.R., Stutes, D., Harrison, M.R. and Tavassoli, M. (1992): Engraftment and long-term expression of human fetal hemopoietic stem cells in sheep following transplantation in utero. *J. Clin. Invest.* **89**:1178-1188.

Zeman, W. and Donahue, S. (1963): Fine structure of the lipid bodies in juvenile amaurotic idiocy. *Acta Neuropathol.* **3**:144-149.

Zeman, W. and Dyken, P. (1969): Neuronal ceroid-lipofuscinosis (Batten's disease): Relationship to amaurotic family idiocy? *Pediatrics* **44**:570-583.

Zeman, W. (1970): Historical development of the nosological concept of amaurotic familial idiocy. In *Handbook of Clinical Neurology,* (Eds. Vinken, P.J. and Bruyn, G.W.). pp. 212-232. North Holland Publishing Co., Amsterdam.

Zeman, W., Donahue, S., Dyken, P. and Green, J. (1970): The neuronal ceroid-lipofuscinoses (Batten-Vogt syndrome). In *Handbook of Clinical Neuorology,* (Eds. Vinken, P.J. and Bruyn, G.W.) pp. 588-679. North Holland Publishing Co., Amsterdam.

Zeman, W. and Siakotos, A.N. (1973): The neuronal ceroid-lipofuscinoses. In *Lysosomes and Storage Diseases.* (Ed. Hers, H.G. and Van Hoof, F.). pp. 519-551. Academic Press, New York.

Zeman, W. (1974): Studies in the neuronal ceroid-lipofuscinoses. *J. Neuropathol. Exp. Neurol.* **33**:1-12.

Zeman, W. (1976): The neuronal ceroid-lipofuscinoses. In *Progress in Neuropathology,* Volume III. (Ed. Zimmerman, H.M.). pp. 203-223. Grune and Stratton, New York.

Zhou, S.Z., Cooper, S., Kang, L.Y., Ruggieri, L., Heimfeld, S., Srivastava, A. and Broxmeyer, H.E. (1994): Adeno-associated virus 2-mediated high efficiency gene transfer into immature and mature subsets of hematopoietic progenitor cells in human umbilical cord blood. *J. Exp. Med.* **179**:1867-1875.