This thesis is dedicated to my darling mama and dada

BIOCHEMICAL STUDIES ON ANIMAL MODELS OF CEROID-LIPOFUSCINOSES

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ABSTRACT

The ceroid-lipofuscinoses are recessively inherited lysosomal storage diseases of children and animals, characterised by brain and retinal atrophy and the accumulation of lipopigment in a variety of cells. A systematic study of isolated lipopigment from an ovine form of the disease had shown the major stored components to be proteinaceous.

This thesis presents further characterisation and identification of the stored ovine lipopigment proteins. Separation of the lipopigment proteins by LDS-PAGE showed the presence of the 3.5 kDa and 14.8 kDa proteins noted in earlier studies, and an additional band at 24 kDa. The 14.8 and 24 kDa bands varied between preparations and from different gels of the same isolate. Radioiodination of lipopigment and silver staining of the proteins separated by LDS-PAGE indicated that the 3.5 kDa protein was the dominant protein component. As these proteins were unable to be separated from each other, exploitation of the molar dominance of the 3.5 kDa protein led to its identification by a non traditional sequencing approach. The major stored protein was shown to be the full proteolipid subunit *c* of the mitochondrial ATP synthase complex. The 14.8 and 24 kDa proteins were shown to be stable oligomers of subunit *c*. Quantitaion of the sequence data showed that subunit *c* accounted for at least 50% of the lipopigment mass. No other mitochondrial protein was detected. Analyses of isolated mitochondria showed that they were functionally normal and did not contain excess amounts of subunit *c*.

Subunit *c* is classified as a proteolipid, due to its lipid-like solubility in chloroform/methanol mixtures. Its storage in lysosome derived lipopigment bodies explained many of the described physical characteristics of lipopigment in the ceroid-lipofuscinoses.

Application of the same methodology showed that a bovine, and two distinct canine forms of the ceroid-lipofuscinoses were also subunit *c* storage diseases.

It is postulated that the lesions in the ceroid-lipofuscinoses involve defects in the degradative pathway of subunit *c* at some point after its incorporation into the inner mitochondrial membrane.

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PUBLICATIONS

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. C., 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(10)** 5736-5740

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.

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

Palmer, D. N., Fearnley, I. M., Medd, S. M., Walker, J. E., Martinus, R. D., Bayliss, S. L., Jolly, R. D., Hall, N. A., Lake, B. D. and Wolfe, L. S. (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. 221-233. Plenum Press, New York.

Jolly, R. D., Martinus, R. D. and Palmer, D. N. (1990). The ovine and other models of ceroid-lipofuscinosis: Their relevance to Batten's disease. *Am. J. Med. Genet.* (In Press).

Martinus, R. D., Harper, P. A. W., Jolly, R. D., Bayliss, S. L., Midwinter, G. G., Shaw, G. J. and Palmer, D. N. (1990). Bovine ceroid-lipofuscinosis (Batten's disease): The major species stored is the DCCD-reactive proteolipid, subunit *c*, of mitochondrial ATP synthase. (Submitted).

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COMMON ABBREVIATIONS USED

ACR	Acceptor control ratio
ADP	Adenosine 5'-phosphate
ATP	Adenosine 5'-triphosphate
ATV	Antibiotic-trypsin-versene
Da	Dalton
DCCD	Dicyclohexylcarbodiimide
EDTA	Ethylenediaminetetra-acetate
FBS	Foetal bovine serum
H&E	Haematoxylin and eosin
HPLC	High pressure liquid chromatography
kDa	kilo Dalton
LDS	Lithium dodecyl sulphate
LDS-PAGE	Lithium dodecyl sulphate polyacrylamide gel electrophoresis
MEM	Minimum essential medium
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PSK	Penicillin, streptomycin and kanamycin
PTH	Phenylthiohydantoin
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TLC	Thin layer chromatography

CHAPTER 1

GENERAL INTRODUCTION

The ceroid-lipofuscinoses are a group of recessively inherited lysosomal storage diseases of children and domestic animals. They are characterised pathologically by brain and retinal atrophy and the presence of fluorescent lipopigment bodies in neurones and a variety of other cell types throughout the body. Clinical features include blindness, seizures, mental retardation and dementia ultimately leading to premature death.

The term ceroid-lipofuscinosis was introduced as a descriptive name for these diseases by Zeman and Dyken (1969) on the basis of similar histochemical and fluorescent characteristics of the stored lipopigment to those of pigments ceroid and lipofuscin. Prior to this, these diseases were grouped with the gangliosidoses as forms of amaurotic familial idiocy. They are presently also known generically as Batten's disease. Lipofuscin (age pigment) and ceroid are two types of intracellular cytosomes (lipopigments) characterised by a yellow-brown colour, fluorescence under ultraviolet light and staining with lipid stains. The former is regarded as a normal age related phenomenon and the latter as a pathological pigment associated with Vitamin E deficiency (Porta and Hartroff, 1969).

There are up to 10 subtypes of ceroid-lipofuscinosis which differ in the age of onset and in the progression of clinical disease. However, the main entities are the infantile (Haltia-Santavuori), late infantile (Jansky-Bielschowsky), juvenile (Batten, Spielmeyer-Sjogren) and an adult (Kufs) form (Lake, 1984; Dyken, 1988; Berkovic, *et al.*, 1988; Boustany, *et al.*, 1988; Wisniewski, *et al.*, 1988). Collectively they are believed to be the most common type of lysosomal storage disease in humans. Prevalence estimates as high as 1 in 25,000 and 1 in 12,500 live births have been reported (Zeman, 1976; Rider and Rider, 1988).

Similar diseases have also been described in a number of domestic animals as follows:

SPECIES	BREED	KEY REFERENCES
Bovine	Beefmaster	Read and Bridges,1969
	Devon	Harper, *et al.*,1988
Canine	English Setter	Koppang,1970;1973/74;1988; Armstrong,1982;Armstrong and Koppang, 1982
	Chihuahua	Rac and Giesecke,1975; Jolly and Hartley,1977
	Dachshund	Cummings and de Lahunta,1977; Vandevelde and Fatzer,1980
	Saluki	Appleby, *et al.*,1982
	Cocker Spaniel	Wilkie and Hudson,1982
	Dalmation	Goebel and Dahme,1985
	Blue Heeler	Cho, *et al.*, 1986; Wood, *et al.*,1987
	Border Collie	Taylor and Farrow, 1988
	Tibetan Terrier	Riis, *et al.*, 1990
Feline	Siamese	Green and Little,1974
Ovine	South Hampshire	Jolly, *et al.*, 1980; 1982
Goat	Nubian	Fiske and Storts,1988

Despite considerable clinical and pathological studies the underlying metabolic bases of the ceroid-lipofuscinoses remain unknown. In storage diseases, the deficient enzyme should be reflected by the nature of the dominantly stored or accumulated chemical species. However, an approach to identify such dominant species has not been widely adopted in research

into the ceroid-lipofuscinoses. This is probably due, in part, to the limited amount of *post mortem* tissue available from the human forms and difficulties encountered in isolating and solubilising the lipopigment.

The lipid staining and fluorescence properties of the stored lipopigment, which had been likened to those of ceroid and lipofuscin (Zeman and Dyken, 1969), implied that their accumulation reflected a similar pathogenesis. This was perceived to be associated with peroxidation of lipid and the formation of Schiff base polymers from malonaldehyde and amino acids, produced during free radical peroxidation of polyunsaturated fatty acids (Chio and Tappel, 1969a,b).

A deficiency in leucocyte peroxidase in late infantile and juvenile forms of the disease was reported by Armstrong, *et al.*, (1973). These findings led Zeman (1974) to suggest that since malonaldehyde could be produced during free radical peroxidation of polyunsaturated fatty acids, the formation of fluorescent lipopigments in the ceroid-lipofuscinoses could involve abnormal peroxidation of lipids. A number of other reports confirmed deficiencies of leucocyte peroxidase in the late infantile (Armstrong, *et al.*, 1974a; Awasthi, *et al.*, 1977; Jensen, *et al.*, 1977), juvenile (Armstrong, *et al.*, 1974b; Gadoth, 1978) and adult forms of disease (Armstrong, *et al.*, 1974b; Bozdech, *et al.*, 1980). In contrast, no evidence for a peroxidase deficiency was found in the infantile (Anzil, *et al.*, 1975; Den Tandt and Martin, 1978; Becker, *et al.*, 1979) and juvenile forms of the disease (Haust, *et al.*, 1976; Den Tandt and Martin, 1978). Isoelectric focussing studies revealed no qualitative or quantitative differences in these enzymes in saliva and parotid gland between normal individuals and patients with juvenile neuronal ceroid-lipofuscinosis (Pilz, *et al.*, 1976a,b; Pilz and Goebel, 1977). Armstrong, (1982) later reported that "patients with generalised ceroid-lipofuscinosis have relatively normal levels of peroxidase if total enzyme is measured, but differ in the intracellular distribution", i.e. a decrease in soluble leucocyte peroxidase activity was accompanied by an increase in insoluble or bound peroxidase activity. Much of the debate concerning the apparent peroxidase deficiency in the ceroid-lipofuscinoses has centred on the methodology of measurement and subcellular distribution of these enzymes.

Spectrophotometric determination of peroxidase activity involved the use of hydrogen peroxide as substrate and p-phenylenediamine as hydrogen donor (Armstrong, *et al.*, 1973). The use of this hydrogen donor was stressed by Armstrong, *et al.*, (1974b) as "when other donors are employed the enzyme deficiency may not be demonstrated". Tsan, *et al.*, (1978), however found the use of p-phenylenediamine to be unsuitable for the study of peroxidases. The subcelluar distribution and enzymology of the leucocyte peroxidases have not been well characterised.

Gutteridge, *et al.*, (1982) reported elevated levels of non-protein bound iron in the cerebrospinal fluid from patients affected with the infantile and juvenile forms of ceroid-lipofuscinosis. As iron is a catalyst of the lipid peroxidation process, an increase in iron levels was linked to a decreased ability of the cerebrospinal fluid to inhibit hydroxyl radical production, a necessary step in lipid peroxidation. A defect in iron metabolism was subsequently suggested as a possible mechanism in the pathogenesis of the ceroid-lipofuscinoses (Gutteridge, *et al.*,1983). However, Heiskala, *et al.*, (1988) reported that concentrations of loosely bound iron and copper in cerebrospinal fluid of patients with infantile, late infantile and juvenile forms of disease did not correlate with clinical diagnosis, nor with the degenerative symptoms of the disease. They concluded that there was no support for a major role for iron toxicity in the development of neuronal degeneration.

The nature of the fluorophore responsible for the fluorescence of stored lipopigment has also attracted much interest and speculation, as it has been considered to be significant to the pathogenesis of the ceroid-lipofuscinoses. Studies of the lipid component of lipopigment showed the presence of a fluorescent acidic structure that concentrated at the origin in normal phospolipid thin layer chromatography developing solvents (Siakotos, *et al.*, 1972; Siakotos and Koppang, 1973; Zeman, 1976). The so called "acidic lipid polymer" or "Schiff base polymer" was thought to be formed by reaction of malonylaldehyde, a product of peroxidation of polyunsaturated fatty acids, with amino groups. Similarity of the fluorescent and chromatographic properties of this material to those generated with *in vitro* peroxidation studies (Elleder, 1981), later added support to this theory. Corrected

excitation-emission spectra by Katz, *et al.*, (1988) showed that lipopigment from Batten's disease tissues emitted in the yellow-orange region (520-540 nm) and not in the blue region (460-480 nm) expected of the "Shiff base polymer". An increase in the absorption spectra at 280 nm was also noted. This was interpreted to be due to "non-fluorescent lipids".

Another fluorescent polymer found in the neutral lipids of lipopigment was designated "polymalonaldehyde" again on the basis of the similarities of its fluorescent and chromatographic properties to *in vitro* generated "polymalonaldehyde" (Siakotos, *et al.*, 1972; Zeman, 1976; Gutteridge, *et al.*, 1977).

Another hypothesis linked to the putative lipid nature of the stored lipopigment implied a primary defect in fatty acid metabolism. This was developed following observations of abnormal fatty acid profiles in the major phospholipids (phosphatidylcholine, phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine) of brain from children affected with the infantile form of disease (Hagberg, *et al.*, 1968; 1974; Svennerholm, *et al.*, 1975; 1987; Svennerholm, 1976). In particular, an increase in 20:4(n-6), a decrease in 22:4(n-6) and 22:6(n-3) was reported. The name "polyunsaturated fatty acid lipidosis" was subsequently proposed to distinguish this disease from the other forms in which the fatty acid changes were not observed. In contrast, Pullarkat, *et al.*, (1982) reported a decrease in the proportions of 22:6(n-3) in grey matter phosphatidylserine in the infantile, late infantile, adult and 'pigment variant' forms, but no changes in the fatty acids of the other phospholipids.

The lipid peroxidation hypothesis provided a rationale for the therapeutic use of antioxidants such as vitamin E, butylated hydroxytoluene or iron-chelating agents such as desferrioxamine (Zeman, 1974; Santavuori and Westermarck, 1984; Santavuori, *et al.*, 1988). However, "antioxidant therapy" has not produced any significant clinical improvement attributed to this form of treatment in patients affected with ceroid-lipofuscinosis (Santavuori, *et al.*, 1988), and the method of treatment remains controversial.

A fluorescent protein complex was left after multiple solvent extractions of lipopigment isolated from brains of late infantile form of disease (Wolfe, *et al.*, 1977). Spectral and chemical analysis of this component led them to suggest that the fluorescence was due to the presence of a retinoid component, possibly complexed to a small peptide. As this component was shown to account for 50% of the dry weight of the storage material, a defect in retinoic acid metabolism was suggested as a possible mechanism of pathogenesis in the ceroid-lipofuscinoses. However, these spectral assignments were ambiguous and have also been shown to be compatible with a cholesterol and/or retinol-cholesterol complex (Nelson and Halley, 1977).

The presence of dolichol, another isoprenoid, at "elevated" levels in brain tissue, isolated storage cytosomes and in the urinary sediment of patients with infantile, late infantile and juvenile forms of disease has been reported (Wolfe, *et al.*, 1977; Ng Ying Kin and Wolfe, 1982; Ng Ying Kin, *et al.*, 1983). As a consequence defects in dolichol metabolism or processing of golgi derived lysosomes and membranes have been suggested as possible causes of the disease. However, similar incorporation of [^{3}H]mevalonolactone and [^{14}C]acetate into dolichol fractions in cultured skin fibroblasts from patients with neuronal ceroid-lipofuscinosis and control fibroblasts led Paton and Poulos, (1984) to conclude that the disease did not involve a defect in dolichol metabolism. Increase in dolichol in urinary sediment led to the suggestion that urine dolichol measurements could be used as a biochemical marker of the disease (Wolfe, *et al.*, 1986). The low specificity of the test due to numerous false positives from bacterial contamination, alcohol ingestion and vigorous exercise prior to the test may limit its usefulness (Wolfe, *et al.*, 1988). It is possible that the urinary dolichol levels reflect the excretion of the storage lipopigment in sloughed renal tubular cell cytoplasm (R. D. Jolly, pers. comm.). Intracellular dolichol is found within lysosomes and is generally regarded as a lysosomal marker (Wong, *et al.*, 1982). Increased dolichol levels in the cerebral cortex of patients with Alzheimer's disease and in aged individuals (Wolfe, *et al.*, 1982; Pullarkat and Reha, 1982; Ng Ying Kin, *et al.*, 1983), suggested that increased dolichol levels in brains of ceroid-lipofuscinosis patients may reflect a secondary phenomenon. Elevated urinary dolichol levels in chronic

alcoholics has also been reported (Pullarkat and Raguthu, 1985).

Significantly higher contents (up to 20 times) of phosphorylated dolichols (P-dolichol) have been reported in whole tissue extracts of patients with ceroid-lipofuscinosis relative to that in age-matched controls (Hall and Patrick, 1985; Pullarkat, *et al.*, 1988; Daniel, 1990). Qualitative analyses indicated that the P-dolichol that accumulated in brains of late infantile and juvenile patients with ceroid-lipofuscinosis was largely linked to oligosaccharides ranging in size from four to fourteen monosaccharide units (Hall and Patrick, 1988). A defect in the metabolism of dolichol-linked oligosaccharides (Dol-PP-OS) involved in the glycosylation of proteins was subsequently suggested as a possible biochemical basis of the disease (Pullarkat, *et al.*, 1988; Pullarkat, 1990). However, no evidence has yet been reported for the presence of any abnormal oligosaccharides or defective glycoproteins in the ceroid-lipofuscinoses. Wolfe, *et al.*, (1988) suggested that an increase in dolichyl phosphates may not be unique to the ceroid-lipofuscinosis, as they were also found to be increased in brains of G_{M1}-gangliosidosis and Tay-Sachs disease patients. As the Dol-PP-OS isolated from lipopigment was estimated to account for 7% or less of the dry weight of lipopigment, (Hall, *et al.*, 1990) their presence in the disease was unlikely to represent the primary biochemical defect.

Ivy, *et al.*, (1984) showed that injections of leupeptin (a thiol-proteinase inhibitor) or chloroquine (a general lysosome enzyme inhibitor) into brains of rats induced lysosome associated granular aggregates resembling the lipopigment found in patients with neuronal ceroid-lipofuscinoses. Accumulation of lipopigment in these diseases by a similar defective (or absent) lysosomal proteinase was suggested. These findings and the observed concommitant increases in dolichol levels in rat brains treated with leupeptin and chloroquine led Wolfe, *et al.*, (1987) to propose a relationship between "dolichols and lysosomal organelle membrane turn over". A specific defect in a cysteine proteinase, important to the recycling and exocytosis of organellar membrane proteins was postulated as a possible cause for the ceroid-lipofuscinoses. As glycosylation of thiol-endoproteases are regulated by the levels of dolichol phosphates in the golgi and endoplasmic reticulm, over-glycosylation due to high levels of dolichol phosphates has also been

suggested as a possible mechanism leading to a putative protease defect (Boustany and Kolodny, 1989).

A variable decrease in cathepsin B activity in fibroblasts from patients with various forms of ceroid-lipofuscinosis has been reported (Dawson and Glaser, 1987). This was considered to be a secondary effect due to accumulation of abnormal peroxides, resulting from a deficiency of a specific phospholipase A_2 (Dawson and Glaser, 1988). However, impaired lysosomal phospholipase A_1 activity and normal activity of phospholipase A_2 in tissues from "some cases" of humans with neuronal ceroid-lipofuscinosis has also been reported (Dawson, 1990). The significance of these results to the disease, if any, remains to be determined.

Eto, *et al.*, (1990) reported that cathepsins B, H, and L activities in cultured skin fibroblasts from patients with the juvenile form of the disease were similar to those determined from control fibroblasts.

Reduction in fasting serum very low density lipoprotein (VLDL) in patients with the juvenile form of the disease, suggested a metabolic defect in lipid transport (Gillis, *et al.*, 1987; Bennett, *et al.*, 1988). Another observation involved a decrease in erythrocyte membrane fluidity in patients with the juvenile form of disease, possibly due to a decrease in docosahexenoic acid (Kohlschutter, *et al.*, 1988).

Immunoreactivity of lipopigments from the juvenile form of the disease was noted with monoclonal antibodies raised against fragments of amyloid β-protein. Localisation of this immunoreactivity to a 31 kDa protein from isolated lipopigment led Kitaguchi, *et al.*, (1990) to suggest that pathogenesis might involve defective processing of amyloid precursor protein from which β-protein is derived. The β-protein is a major component of amyloid deposits in aging and Alzheimer's disease and is thought to be deposited as a result of a proteolytic processing defect of the precursor protein (Dyrks, *et al.*, 1988). However, as the 31 kDa protein isolated from lipopigment has not yet been characterised, the significance of this result to the ceroid-lipofuscinoses remains to be determined.

Most of the above experimental observations and hypotheses have been based on the lipid staining and fluorescence characteristics of the stored lipopigment, and compounds found at "elevated levels" in total tissue extracts. None have led to the elucidation of the underlying biochemical anomaly in the ceroid-lipofuscinoses.

Ceroid-lipofuscinosis has been recorded in a number of animal species as noted earlier. However, most of these reports are case studies and the diseases have not been fully characterised. The disease in the English Setter dog though, is one that has been studied clinically and pathologically as a model of the juvenile human disease (Koppang, 1970; 1973/74; 1988; Goebel, *et al.*, 1982).

Biochemical investigations into the canine disease have centered on peroxidase enzymes and fatty acid abnormalities (Patel, *et al.*, 1974; Siakotos, *et al.*, 1978; Armstrong, *et al.*, 1978a,b; Armstrong, 1982; Armstrong and Koppang, 1982; Farnsworth, *et al.*, 1982; Keller, *et al.*, 1984; Reddy, *et al.*, 1985; Koppang, 1988). These studies have been linked with the putative lipid peroxidation theories of pathogenesis in the ceroid-lipofuscinoses.

The disease in the South Hampshire sheep has also been extensively studied as a model of the juvenile human disease (Jolly, *et al.*, 1980; 1982; 1988; 1989; Graydon and Jolly, 1984; Mayhew, *et al.*, 1985). The use of such an animal model has greatly advanced the study of the ceroid-lipofuscinoses. Not only is experimental material readily available but the ability to euthanase an animal and commence isolation of lipopigment bodies within minutes of death has the advantage that artefactual *post mortem* changes can be minimised.

The analogous disease in sheep shows retinal degeneration and severe brain atrophy. The latter is a distinctive feature of the ceroid-lipofuscinoses relative to other lysosomal storage diseases. Although the brains of new born affected lambs were within the normal weight range for the first 4 months, by terminal disease at 24-26 months of age, brain weights were 50% of those of normal sheep at that age (Mayhew, *et al.*, 1985; Jolly, *et al.*,

1989). Atrophy began with a laminar necrosis in the parietal area of the cerebral cortex becoming more diffuse and spreading to the occipital and lastly the temporal lobes. It was accompanied by an increasingly severe fibrillary astrocytosis and an increase in lipopigment-laden macrophages. Although storage of physically similar lipopigment occurs in a variety of other lysosomal storage diseases and in normal aging, neuronal necrosis is not such a feature. This suggested that in ceroid-lipofuscinosis, neuronal necrosis was associated with the metabolic lesion rather than storage of lipopigment *per se* (Jolly, *et al.*, 1989; 1990).

Lipopigment stored in neurones stains with Sudan black, luxol fast blue and periodic acid-Schiff (PAS) stains. They also have a yellow fluorescence when irradiated with 366nm uv light in both paraffin and frozen sections. Intensity of fluorescence and staining increases with age reflecting increasing size and total amounts of the stored lipopigment. Storage material is also found in cardiac muscle, hepatocytes and Kupffer cells, kidney and pancreatic epithelial cells and a wide variety of other cells within the body (Jolly, *et al.*, 1980; 1982). Ultrastructurally they appear as electron dense, membrane-bound, irregularly rounded bodies. They have a granular texture but also many lamellar profiles which have been described as fingerprint, curvilinear, crystalloid or multilamellar structures. The reason for this diversity of structures is not yet understood. They have however, been interpreted as complex, three dimensional matrices in which repeating units of protein and lipid form paracrystalline structures as a result of the condensation of bilayer membranes (Jolly, *et al.*, 1988; 1989). Their freeze fracture electron microscopic appearance and powder X-ray diffraction pattern is thought to support this interpretation (Jolly, *et al.*, 1988).

The primary role of peroxidation of polyunsaturated fatty acids in lipopigment formation in ceroid-lipofuscinosis was questioned by Palmer, *et al.*, (1985). This was on the basis of the similarity of the brain grey matter phospholipid fatty acid compositions of sheep affected with ovine ceroid-lipofuscinosis to those of control sheep. There were no signs of essential fatty acid deficiency in the affected sheep. Sheep as ruminants must conserve their restricted fatty acids for structural functions, and as such, if there was a fatty acid defect, it should have been reflected in the phospholipid fatty acid compositions. It was concluded that an abnormality in fatty acid metabolism

was not primarily involved in the pathogenesis of the disease.

A relatively simple method of lipopigment isolation was developed by Palmer, *et al.*, (1986a). This enabled the systematic characterisation of the stored lipopigment. Lipopigment isolated from liver, kidney, pancreas and brain of affected sheep was shown to contain 16-27% lipid. Phospholipids accounted for 50% of liver lipopigment lipids and contained the normal mammalian membrane components phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and bis(monoacylglycero)phosphate, a known lysosomal lipid component (Bleistein, *et al.*, 1980). The remaining 50% of the lipid fraction was accounted for by the neutral lipids cholesterol, dolichol, dolichyl esters and ubiquinone. These were thought to be indicative of a lysosomal origin of the lipopigment. Dolichol, in the form of dolichol pyrophosphate linked oligosaccharides, was also present accounting for 0.1-2% of the total lipopigment mass (Hall, *et al.*,1989). The concentrations and distributions of lipopigment metals accounting for 1-2% (copper, iron, calcium, zinc, manganese, aluminum, nickel and chromium) were also considered consistent with the lipopigment cytosomes functioning as lysosomes at some stage in their biogenesis (Palmer, *et al.*, 1988).

The major component of isolated lipopigment (65-75%) was found to be proteinaceous in nature. This material was difficult to handle due to its relative insolubility. Of the large range of solvent systems tried, only SDS in the presence of 5% 2-mercaptoethanol and formic acid were able to dissolve the lipopigment or delipidated protein obtained from it (Palmer, *et al.*, 1986b). Silver staining of lipopigment proteins separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed two major protein bands, one that ran to the lower region of the gel with an apparent molecular weight of 3.5 kDa and another at 14.8 kDa. These proteins were relatively insensitive to Coomassie blue stains. The 3.5 kDa lipopigment band was present in such amounts that its abnormal accumulation allowed the distinction to be made between total control and affected whole tissue homogenates, a classic criterion for the identification of the abnormally stored components in lysosomal storage diseases.

The analyses by Palmer, *et al*, (1986a,b) failed to show any products of lipid

peroxidation or abnormality in the stored lipids, nor was there a depletion of unsaturated fatty acids. No quantitatively dominant fluorophore was found in the lipid fractions. In fact, the fluorescence of the bodies was lost on separation of the various components. There was no evidence to suggest that disturbances in metal metabolism led to lipopigment formation. The small amounts of phosphorylated dolichol (0.2-2%) also indicated that a defect in metabolism of dolichol-linked oligosaccharides was unlikely to reflect the primary cause of the ovine disease (Hall, *et al.*, 1989). The presence of low molecular weight proteins as the major lipopigment component led Palmer, *et al.*, (1986a,b) to conclude that the ovine disease was not a lipidosis but represented a lysosomal proteinosis.

As the ovine disease was considered to be a good model of the juvenile human disease, these findings represented a considerable advance in the understanding of the ceroid-lipofuscinoses.

A number of mechanisms that could lead to a lysosomal proteinosis were proposed by Jolly, *et al.*, (1988). These were a deficiency of a lysosomal protease or its control, secondary perturbation of lysosomal function similar to that induced by iatrogenic drugs or NH_4Cl, the presentation to the lysosomal system of post-translationally modified protein that cannot be catabolised, the presentation of protein in a complex that protected it from proteolysis, or a defect in recycling from lysosomes of some specific membrane domain.

This thesis describes the identification of the major stored ovine lipopigment protein, considered to be specific to the disease. The relative insolubility and difficulty in separation of the lipopigment protein resulted in a non-traditional approach being adopted in its subsequent characterisation. Application of the methodology developed during studies on the ovine lipopigment was also used to define the major stored lipopigment component from two canine forms and a bovine case of ceroid-lipofuscinosis.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 ANIMALS AND TISSUES

Sheep with ceroid-lipofuscinosis used in this study were from an inbred flock of South Hampshires, maintained by the mating of heterozygous ewes with 7-8 months old homozygously affected rams. These animals were husbanded at Massey University under standard New Zealand pasture farming conditions. Diagnosis of affected lambs was established by histopathology of brain biopsies at 2.5-3 months of age (Dickson, *et al.*, 1989). Tissues for experimentation were obtained at autopsy of affected sheep 12-24 months old, and placed in ice-cold 0.01 M phosphate buffered saline (PBS), pH 7.2 within minutes of euthanasia by barbiturate anaesthesia and exsanguination. Confirmation of the earlier diagnosis of ceroid-lipofuscinosis was made from gross evidence of brain atrophy and/or histopathology. Control tissues were obtained from age matched normal Southdown or New Zealand Romney sheep.

2.2 ISOLATION OF LIPOPIGMENT

Lipopigment was isolated from pancreas, kidney, brain and liver of affected sheep by a combination of homogenization, osmotic lysis, sonication and centrifugation (Palmer, *et al.*, 1988).

From Pancreas, Liver and Brain

Pancreas, liver or brain grey matter (2-40 g), was rinsed with PBS and homogenized in 10 vol of ice-cold 0.4 mM Tris-HCl, pH 7.4 for 1 min in a Sorvall Omnimixer (Ivan Sorvall Inc.). The homogenate was filtered through gauze, sonicated for 1 min, filtered through glass wool, then pelleted in a Sorvall GLC-1 centrifuge for 30 min at 1,400 g_{max}. The pellet was resuspended in deionized water and subjected to centrifugation at 12,000 g_{max} for 20 min. A white fluffy layer was gently washed off the pellet which was then resuspended in deionized water, and pelleted by centrifugation at 12,000 g_{max} for 20 min. This procedure of washing and centrifugation was

repeated until a uniform pellet was obtained. The final pellet was suspended in deionized water or dissolved in 1% lithium dodecyl sulphate (LDS) and stored at -20°C until required.

From Kidney

Lipopigment bodies from kidney cortical tissue were isolated using the same method as above, except that a soft brown pellet was removed from the sonicated homogenate by sedimentation at 70 g_{max} in a Sorvall GLC-1 centrifuge for 5 min.

2.3 THIN SECTION ELECTRON MICROSCOPY

Aliquots of isolated lipopigment were fixed in 2% glutaraldehyde and 3% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2, post fixed in 1% osmium tetroxide and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined in a Phillips 200 transmission electron microscope.

2.4 AMINO ACID ANALYSIS

Samples were subjected to hydrolysis *in vacuo* in 6 M glass distilled HCl containing 1% phenol for 24 h at 110°C. The hydrolysates were dried *in vacuo* over NaOH pellets and the amino acids analysed on a Beckman 119L amino acid analyzer. Cystine content was determined in some samples by analysis of cysteic acid after oxidation with performic acid (Hirs, 1967). Protein concentrations were calculated from this data and also by the method of Lowry, *et al.*, (1951). The samples analysed were (a) proteins precipitated from solutions of lipopigments dissolved in 1% LDS with cold acetone, (b) ether precipitated proteolipids extracted from lipopigment dissolved in chloroform/methanol (2:1, v/v) containing 100 mM ammonium acetate, (c) the nonextracted material that precipitated at the interface during the modified Folch extraction, and (d) lipopigment bodies suspended in deionized water that were dried to constant weight *in vacuo* over NaOH pellets.

2.5 LITHIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (LDS-PAGE)

LDS-15% and 20% polyacrylamide gels, (1.5 mm x 10 cm x 16 cm), were prepared in a Protean Dual Slab Cell (Bio-Rad, Richmond, Ca) by the method of Laemmli (1970), except that the *bis*acrylamide to acrylamide ratio was 1:29 w/w instead of 1:37.5 w/w, and 10% w/w sucrose was added to the main gel (Palmer, *et al.*, 1986b).

Fresh and frozen isolated lipopigment proteins dissolved in 1% LDS at 2 mg protein/ml were diluted with deionized water and added to a solution of boiled glycerol and bromophenol blue to a final concentration of 12% glycerol and 6 μg/ml of bromophenol blue. Lipopigment containing 15-25 μg of protein in 50 μl was loaded onto each lane of the gel. Samples containing the lipopigment proteins were not heated at any stage. Electrophoresis was carried out at a constant current, 8 mA, at 4°C, until the bromophenol blue reached the bottom of the gel, in approximately 17 h. Apparent molecular weights of the proteins were determined by comparison of their migration rates with those of molecular weight standards obtained from Sigma (Kit No. MW-SDS 70L).

2.6 SILVER STAINING OF POLYACRYLAMIDE GELS

After electrophoresis the gels were fixed for 60 min in 200 ml of 12% (w/v) trichloroacetic acid. They were then washed for 60 min with 800 ml of 40% methanol containing 10% acetic acid, twice for 30 min with 400 ml of 10% ethanol containing 5% acetic acid and finally for 10 min with a 200 ml solution of 3.4 mM potassium dichromate containing 3.2 mM nitric acid. After removal of all the oxidizing agent from the gels by washing with deionized water, they were stained for 10 min with 200 ml of 12 mM silver nitrate, rinsed for 2 min with deionized water and developed by successive additions of portions of a solution of 0.28 M sodium carbonate containing 6.33 mM formaldehyde (0.019%). The first development was for 1 min, the second for 5 min and the third was continued until the desired staining intensity was reached. Development was stopped by washing the gels for 5 min with 400 ml of 5% (v/v) acetic acid. All steps after the addition of the

silver nitrate solution were carried out under a photographic safe light (Kodak, Wratten series filter OB). Gels were then destained for 2 min with 200 ml of a solution of 30 mM potassium ferricyanide and 65 mM sodium thiosulphate (Farmer's reducer). After complete removal of this reagent by washing with deionized water, the gels were stained again with 12 mM silver nitrate, and developed as before.

2.7 CHEMICALS

Lithium dodecyl sulphate (LDS), 2-mercaptoethanol, bovine serum albumin (BSA), Iodogen (1,3,4,6-tetrachloro 3α,6α-diphenylglycoluril), Coomassie Brilliant Blue R-250, (3-[Cyclohexylamino]-1-propanesulfonic acid) (CAPS) cyanogen bromide and molecular weight standards (Kit No. MW-SDS 70 L) were obtained from Sigma (St. Louis, MO. U.S.A.). All chemicals required for silver staining were obtained from Bio-Rad (Richmond, CA. U.S.A.) except for sucrose and trichloroacetic acid (TCA) which were obtained from BDH (Poole, England). The silver nitrate was obtained from Ajax chemicals (N.S.W., Australia). The 2,5-diphenyloxazole (PPO) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP)were also obtained from BDH. Adenosine 5'-diphosphate, disodium (ADP) cat No. 10490 was obtained from United States Biochemical Corporation (Cleveland, Ohio, U.S.A.). Sephadex G-25 fine grade, was obtained from Pharmacia Fine chemicals (Uppsala, Sweden). Carrier free Na ^{125}I in NaOH (100 mCi/ml), NCS tissue solubiliser and ^{3}H-amino acid mixture (code TRK.440) (1.0 mCi/ml) were obtained from Amersham International P.L.C. (Amersham, Bucks, England). Polyvinylidene difluoride membranes (PVDF) were obtained from Millipore (Millipore Corp. Bedford, MA. U.S.A.). DC-Plastikofolien cellulose TLC sheets were from Merck (E. Merck, Darmstadt, West Germany). Eagles minimum essential media (MEM) (Cat. No. 10-101) and MEM vitamins (Cat. No. 16-014-49) were from Flow Laboratories, Inc. (West Germany). Trypsin, foetal bovine serum (FBS) and tryptose broth were obtained from Difco Laboratories (Detroit, MI, U.S.A). Plastic 25cm^2 tissue culture flasks were from Nunclon, InterMed (Denmark) andLeighton tubes were from Kimax (U.S.A.). All water was purified through a Milli-Q Reagent water system and a Millistak GS filter from Millipore, so that it had a minimum resistance of 10 M ohms/cm. All other reagents were of an analytical grade and all solvents used were double distilled.

CHAPTER 3

LDS-PAGE BEHAVIOUR AND ^{125}I RADIOLABELLING OF PANCREATIC LIPOPIGMENT PROTEINS.

3.1 INTRODUCTION.

Analysis of lipopigment isolated from pancreas, liver, kidney and brain of sheep affected with ceroid-lipofuscinosis has shown that 70% of the lipopigment mass was protein, most of the remainder being neutral lipids and phospholipids expected for the lysosome derived lipopigment bodies. Isolated lipopigment could be solubilised by sodium dodecyl sulphate only in the presence of 5% 2-mercaptoethanol. Subsequent separation of the lipopigment proteins by SDS-PAGE, showed a major band migrating with an apparent molecular weight of 3.5 kDa at the limit of resolution of the gel, heterogeneous material between 5.0-9.0 kDa and a band at 14.8 kDa (Palmer, *et al.*, 1986b). The PAGE behaviour of these specific lipopigment proteins was further investigated. The presence of the 3.5 kDa band in affected but not in control tissue homogenates indicated that it was specific to the disease. Its relative silver staining intensity implied that it was a major component of the isolated lipopigment. The silver stain could not however be taken as a quantitative measure of these proteins due to the fact that differential sensitivity of different proteins to stains is well documented (Friedman, 1982; Merril, 1986).

The 14.8 and 3.5 kDa lipopigment bands extracted from polyacrylamide gels were shown to contain similar relative amounts of tyrosine (S.M. Cooper, pers. comm.). Radioiodination of pancreatic lipopigment proteins was therefore investigated as a means of measuring them quantitatively and as a method of obtaining a radiolabelled substrate.

3.2 SPECIAL MATERIALS AND METHODS

3.2.1 ^{125}I RADIOLABELLING OF PANCREATIC LIPOPIGMENT PROTEINS

An Iodogen (1,3,4,6-tetrachloro 3α, 6α-diphenylglycoluril) solution was prepared by the method of Markwell and Fox (1978). Iodogen was dissolved in dichloromethane, (40 μg/ml), 100, 200, 300, 500, 700 μl and 1ml of this solution were added to glass vials and evaporated to dryness under nitrogen at 25°C. Removal of the dichloromethane produced a film of Iodogen on the glass vials. A 10 μl solution of 0.05 M ammonium acetate, pH 7.4, was added to the vials followed by 25 μCi of Na^{125}I. After the addition of 200 μl of pancreatic lipopigment dissolved in 1% LDS (1.5 mg/ml), the reactants were incubated at 25°C for 1, 2, 5, 10, 15, 20 and 30 min. Care was taken to ensure that the reactants were in contact with the Iodogen film on the vials. The iodinations were terminated by transfering the reactants to 500 μl of 0.1% LDS and leaving them for a further 10 min to allow the unincorporated iodous ions to return to molecular iodine. Incorporation of ^{125}I into lipopigment was measured by the method described below. The mixture was applied to a Sephadex G-25 column (3.8 x 200 mm) that had been pretreated by the elution of 1 ml of bovine serum albumin 3% (w/v) in 0.1% LDS to prevent the non-specific absorption of the radiolabelled protein. The first eluted radioactive peak was collected and the protein precipitated by acetone (10:1, v/v). The precipitated protein was re-solubilised in 1% LDS and added to unlabelled pancreatic lipopigment protein for further experimentation.

3.2.2 DETECTION OF THE RADIOLABEL

Incorporation of ^{125}I was determined by TLC. A 10 μl aliquot of the labelled lipopigment was spotted onto DC-Plastikfolien cellulose TLC strips (1 x 14 cm) and developed in trichloroacetic acid 12% (w/v). The distribution of the radioactivity was determined by cutting the strips into 1 cm pieces and counting the gamma emissions on a Nuclear Enterprise NE 1600 gamma counter. The protein remained at the origin and the free iodine (^{125}I) chromatographed with the solvent front (Salacinski, *et al.*, 1981). Proteins in samples of iodinated lipopigment were also separated by LDS-PAGE. The distribution of radioactivity in polyacrylamide gels was determined by cutting the gels into strips, counting the gamma emissions as above, and comparing the count distribution with silver stained side strips.

3.3 RESULTS

3.3.1 LDS-PAGE behaviour of lipopigment proteins

Solubility of lipopigments in LDS in the absence of 2-mercaptoethanol allowed the PAGE behaviour of the lipopigment proteins to be further investigated. Pancreatic lipopigment proteins, separated by LDS-PAGE, were characterised by a major band with an apparent molecular weight of 3.5 kDa. Other bands at 14.8 and 24 kDa were also noted and considered specific to the lipopigment. However, the proportions of the latter two bands varied between preparations and from different gels of the same isolate (Fig. 3.1,A and Fig.4.2). The presence or absence of 5% 2-mecaptoethanol in the sample had no influence on the pattern of the major lipopigment bands obtained (Fig. 3.1, B).

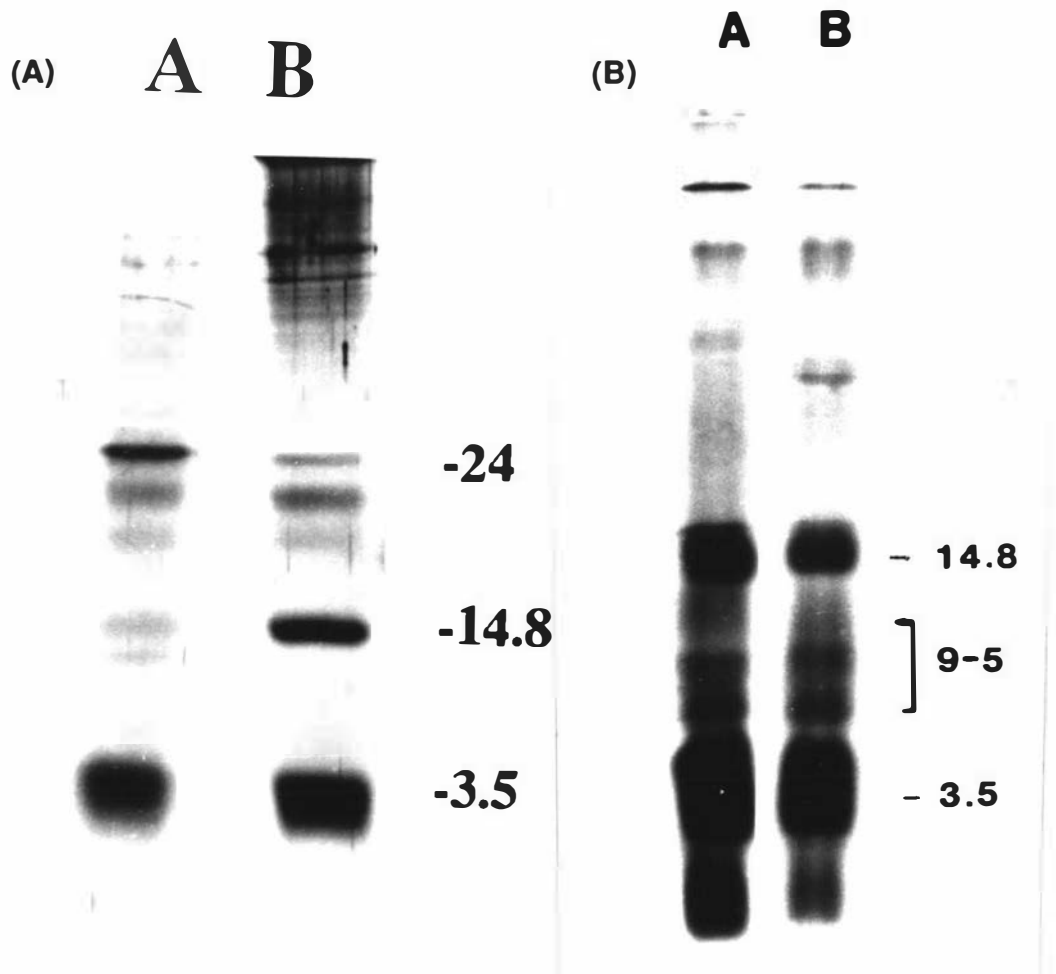

Fig 3.1 A,B Silver stained LDS-20% PAGE of (A) pancreatic lipopigment protein (lane A) and the same sample run under identical conditions on another gel (lane B), (15 µg/lane). (B) Pancreatic lipopigment protein in the presence (lane A) and absence (lane B), of 2-mercaptoethanol, (25 µg/lane). The numbers on the right hand side indicate apparent molecular weights calculated from the migration of molecular weight markers.

3.3.2 Incorporation of ^{125}I into lipopigment proteins

Incorporation of radioactive iodine into lipopigment protein was optimal at 12 µg of Iodogen/vial (Fig. 3.2 A). At this concentration, a time course experiment showed that 10 min was the minimum time required to obtain a maximum incorporation of 70% of the radioactive iodine into the lipopigment proteins at 25°C (Fig. 3.2 B).

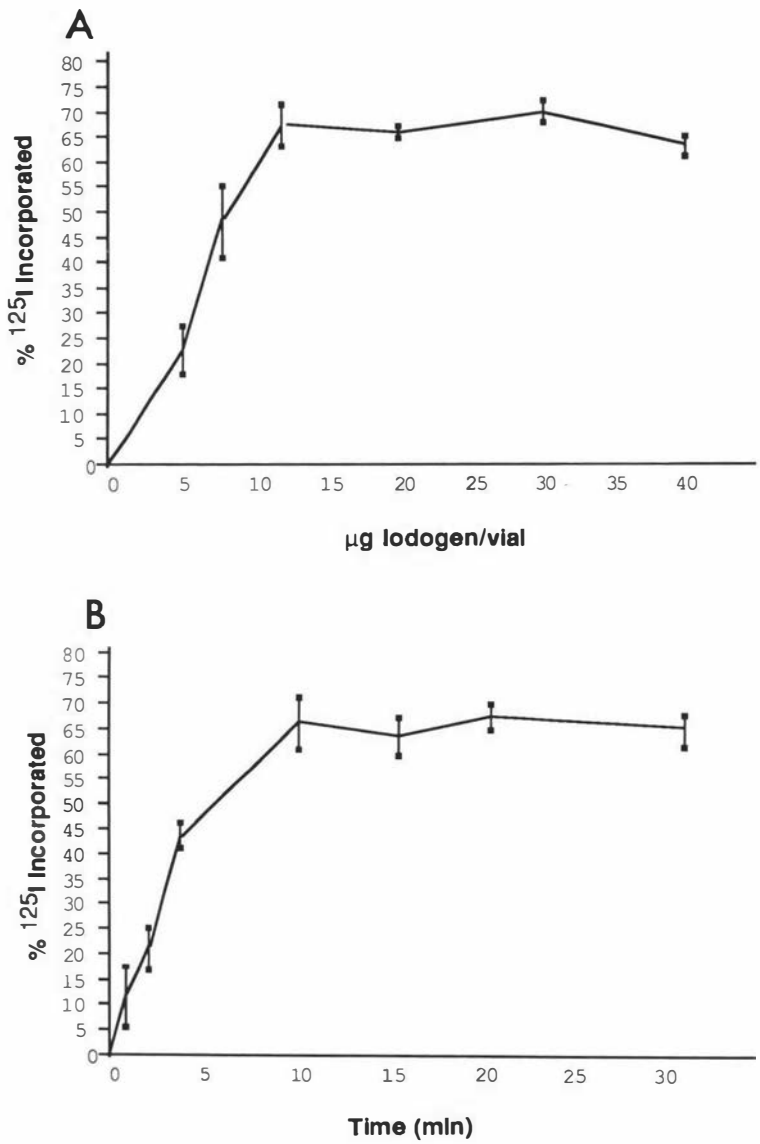

Fig.3.2 A,B Incorporation of ^{125}I at various Iodogen concentrations, (A) and the incorporation of ^{125}I at 12 µg Iodogen/vial at various time intervals, (B). Iodinations were carried out using total pancreatic lipopigment dissolved in 1% LDS at (1.5 mg/ml) and 25 µCi Na ^{125}I, incorporation was determined by the dpm that remained at the origin of the TLC strips compared to the total dpm applied. Each point on the graphs represents individual estimations.

Freshly iodinated pancreatic lipopigment proteins separated by LDS-PAGE showed a similar distribution of the 3.5, 14.8 and 24 kDa lipopigment proteins to that of non-iodinated lipopigment (Fig.3.3, lane A and Fig.3.1A, respectively). This indicated that the iodination procedure had no influence on the subsequent separation of these proteins. However, on storage of the labelled lipopigment for periods longer than a week, aggregation at the stacking gel/main gel interface and smearing along the gels were sometimes observed. With some preparations the complete absence of the 3.5 kDa lipopigment protein was noted (Fig. 3.3, lane B).

Fig.3.3 Silver stained LDS-20% PAGE of ^{125}I radiolabelled pancreatic lipopigment proteins (lane A). An aliquot of the same sample after it had been stored at -20°C for a period of one week (lane B). The numbers on the right hand side indicate apparent molecular weights calculated from the migration of molecular weight markers.

Distribution of ^{125}I amongst freshly radiolabelled pancreatic lipopigment proteins separated by LDS-PAGE showed that the 3.5 kDa lipopigment band contained on average 35% of the total label that entered the gels with the 14.8 kDa band accounting for 22% and the material between the 14.8 and 3.5 kDa bands containing a further 10% of the label (Table 3.1.).

Table 3.1 The distribution of ^{125}I amongst radiolabelled pancreatic lipopigment proteins

Polypeptide bands	% of ^{125}I[a]
above 14.8 kDa	32.8±3.8
14.8 kDa	21.8±0.5
14.8-3.5 kDa	10.1±3.2
3.5 kDa	35.3±6.3

a. Percentage of the total count that entered the LDS-20% polyacrylamide gel ±s.e.m. for three separate estimations. Between 80-95% of the freshly iodinated lipopigment proteins were shown to enter the gels as determined by the recovered radioactivity.

3.4 DISCUSSION

Lipopigment bodies can be isolated from pancreatic tissue relatively free from contamination with other subcellular organelles and debris. Lipopigment proteins from this tissue also give the best separation on polyacrylamide gel electrophoresis. For these reasons pancreatic lipopigment was chosen for the LDS-PAGE and radiolabelling studies.

The similar electrophoretic patterns obtained when total lipopigment protein dissolved in 1% LDS in the presence or absence of 2-mecaptoethanol indicated that the 3.5 kDa and 14.8 kDa lipopigment proteins were not subunits of a larger aggregate held together by intermolecular cystine disulphide bonds. However, the variability of the 24 and 14.8 kDa bands between different preparations and from different gels of the same isolate suggested some form of aggregation that was resistant to dodecyl sulphate and reducing conditions.

The replacement of aromatic hydrogen by electrophilic iodine in activated aromatic systems (tyrosine, histidine, tryptophan) is widely used to label proteins. Electrophilic iodine can be generated by a variety of oxidizing agents: chloramine-T (Greenwood and Hunter, 1963), hydrogen peroxide (Hubbard and Cohn, 1972), chlorine gas (Butt, 1972), and sodium hypochlorite (Redshaw and Lynch, 1974). These form the basis of most available methods of radio-iodination. However, the use of oxidizing agents that come into contact with proteins in solution can lead to oxidative damage to the proteins. Lactoperoxidase and lactoperoxidase-catalyzed iodinations using hydrogen peroxide alone or generated by glucose oxidase systems require the addition of extraneous protein to the system (Marchalonis, 1969). This extraneous protein which can itself become highly labelled during the iodination process, provides an additional complication. An alternative method employs conjugation labelling where N-succinimidyl propionate labelled with iodine is attached covalently to lysine residues or to the amino-terminus of the protein (Bolton and Hunter, 1973). Although this technique offers some advantages over the chloramine-T and lactoperoxidase methods, it is a very tedious method of iodination. The introduction of 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen) as an iodinating

reagent for soluble and membrane proteins provided a method of protein iodination under mild conditions, without sacrificing cellular integrity (Markwell, and Fox, 1978). As the Iodogen method was technically simpler to use, and because it did not require the addition of extraneous protein or strong oxidants to initiate the reaction, nor a strong reducing reagent to terminate it, it was chosen as the method for the radioiodination of pancreatic lipopigment proteins.

It was reasoned that since LDS is a denaturing detergent, ^{125}I radiolabelling of polypeptides in it should incorporate iodine equally into all tyrosine residues. Preliminary amino acid analysis indicated that the 14.8 and 3.5 kDa lipopigment bands contained similar molar ratios of tyrosine residues (S.M. Cooper, pers. comm.). Therefore, the distribution of radiolabel amongst total pancreatic lipopigment proteins separated by LDS-PAGE should provide at least a semi-quantitative estimate of these polypeptides. The 14.8 and 3.5 kDa ovine lipopigment protein bands together accounted for 67% of the radioactive label that entered the LDS-PAGE (Table 3.1), suggesting that they were the dominantly stored chemical species in ovine ceroid-lipofuscinosis. The results also showed that since the 3.5 kDa protein band is the major contributor to the lipopigment protein mass, and is of low molecular weight, it is present in total lipopigment in a higher molar ratio than any other component. A value of 71 moles % was calculated from the radioiodination data presented by assuming an average molecular weight of 50 kDa for the lipopigment proteins above 14.8 kDa and of 5.0 kDa for those between the 14.8 and 3.5 kDa lipopigment bands. Exploitation of this molar dominance enabled the identification of the 3.5 kDa lipopigment protein by quantitative amino acid sequence analysis, which is described in the next chapter. The loss of the 3.5 kDa protein, which was considered to be the major lipopigment protein, on storage of the labelled lipopigment (Fig.3.3, lane B) suggested that the radioiodination of lipopigment was not a suitable method of obtaining a labelled substrate.

CHAPTER 4

IDENTIFICATION OF THE 3.5 kDa LIPOPIGMENT BAND

4.1 INTRODUCTION

Methods available for separation and further characterisation of lipopigment proteins have been limited by the solvents that were able to dissolve them. Only formic acid, 1% SDS in the presence of 5% 2-mercaptoethanol (Palmer, *et al.*, 1986b) and 1% LDS in the presence or absence of 2-mercaptoethanol (Chapter 3) dissolved the lipopigment proteins. Solubility in the latter solutions enabled separation of these proteins by polyacrylamide gel electrophoresis which showed a major band at 3.5 kDa and bands at 14.8 and 24 kDa. These components were however unable to be separated by gel filtration chromatography on Bio-Gel P-10 eluted with formic acid (D.N. Palmer, pers. comm.). Results presented in the previous chapter showed that the 3.5 kDa band was not a subunit of a larger aggregate held together by cystine disulphide bonds. It was also shown to be the major contributor to the total lipopigment protein mass. Separation of the 3.5 kDa component from the other lipopigment proteins by size exclusion high pressure liquid chromatography (HPLC) was investigated.

4.2 SPECIAL MATERIALS AND METHODS

4.2.1 SIZE EXCLUSION HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

Lipopigment protein was obtained from total lipopigment dissolved in 1% LDS by acetone precipitation in 20 vol of cold acetone. After washing with a further 10 vol of cold acetone and with deionized water, the precipitate was redissolved in 1% LDS, filtered through a 0.4 micron filter (Millipore) and 1-2 mg aliquots were injected onto either a TSK G2000 SW (7.5 x 200 mm) or TSK G2000 and G4000 SW HPLC columns connected in series (Toyo Soda manufacturing Co. Tokyo) with a guard column packed with 'Protein I-125' (Waters Associates, Milford MA). The columns were eluted with 0.1% LDS

at a flow rate of 0.6 ml/min. Detection was at 280 nm using a Model 450 Variable Wavelength Detector (Waters Associates, Milford MA). The eluted peaks were collected and freeze dried. After redissolving in deionized water aliquots were analysed by LDS-PAGE as described in section 2.5 and the rest stored at -20°C until required.

4.2.2 AMINO ACID SEQUENCING

Fractions eluted from the HPLC columns that contained the lipopigment proteins were precipitated with 20 vol of cold acetone. After washing with a further 10 vol of cold acetone, samples were dissolved in distilled 98% formic acid at a protein concentration of 50 µg/ml, and aliquots, 50-100 µl, were loaded onto polybrene treated glass filter disks that had been through three cycles of a standard ABI program 03RPRE (Applied Biosystems Inc., Foster city CA. U.S.A). Sequencing was done on an Applied Biosystems 470A gas phase sequencer coupled to a 120A PTH analyzer using a standard ABI program 03RPTH. Aliquots of precipitated protein were also subjected to quantitative amino acid analysis as described in section 2.4 to allow an estimation of the total protein loaded.

4.2.3 REPETITIVE AND INITIAL YIELD CALCULATIONS

Yields of the phenylthiohydantoin (PTH) amino acids produced in the Edman degradation at each cycle were calculated from comparison with freshly prepared PTH amino acid standards. Repetitive yields were calculated from linear regression best fit plots of the logarithms of the yields at each cycle. Initial yields were read from the intercept of the repetitive yield slopes with the y-axis.

4.3 RESULTS

4.3.1 Size exclusion HPLC of lipopigment proteins

Total lipopigment eluted as a number of 280 nm absorbing peaks on size exclusion HPLC using a TSK G2000 SW column run in 0.1% LDS (Fig. 4.1 A). When pooled fractions from these peaks were examined by LDS-PAGE, the peak and shoulder that eluted at 30 min at the size exclusion limit of 50 kDa, was shown to contain all the specific lipopigment proteins in a

distribution similar to that found in total lipopigment. Extending the size range of the separation by connecting the TSK G2000 SW column in series with a TSK G4000 SW column made no difference to this result and all the specific lipopigment proteins eluted between 60-90 min at a size exclusion limit of 400 kDa (Fig. 4.1 B,C). Although the molar ratios of the 14.8 and 3.5 kDa proteins varied from fractions a-c, they were unable to be purified (Fig. 4.1 C). The presence of 100 mM ammonium acetate in the 0.1% LDS buffer was able to move the elution of the lipopigment proteins along the size range of the columns. They were, however, unable to be separated from each other by size exclusion HPLC in LDS.

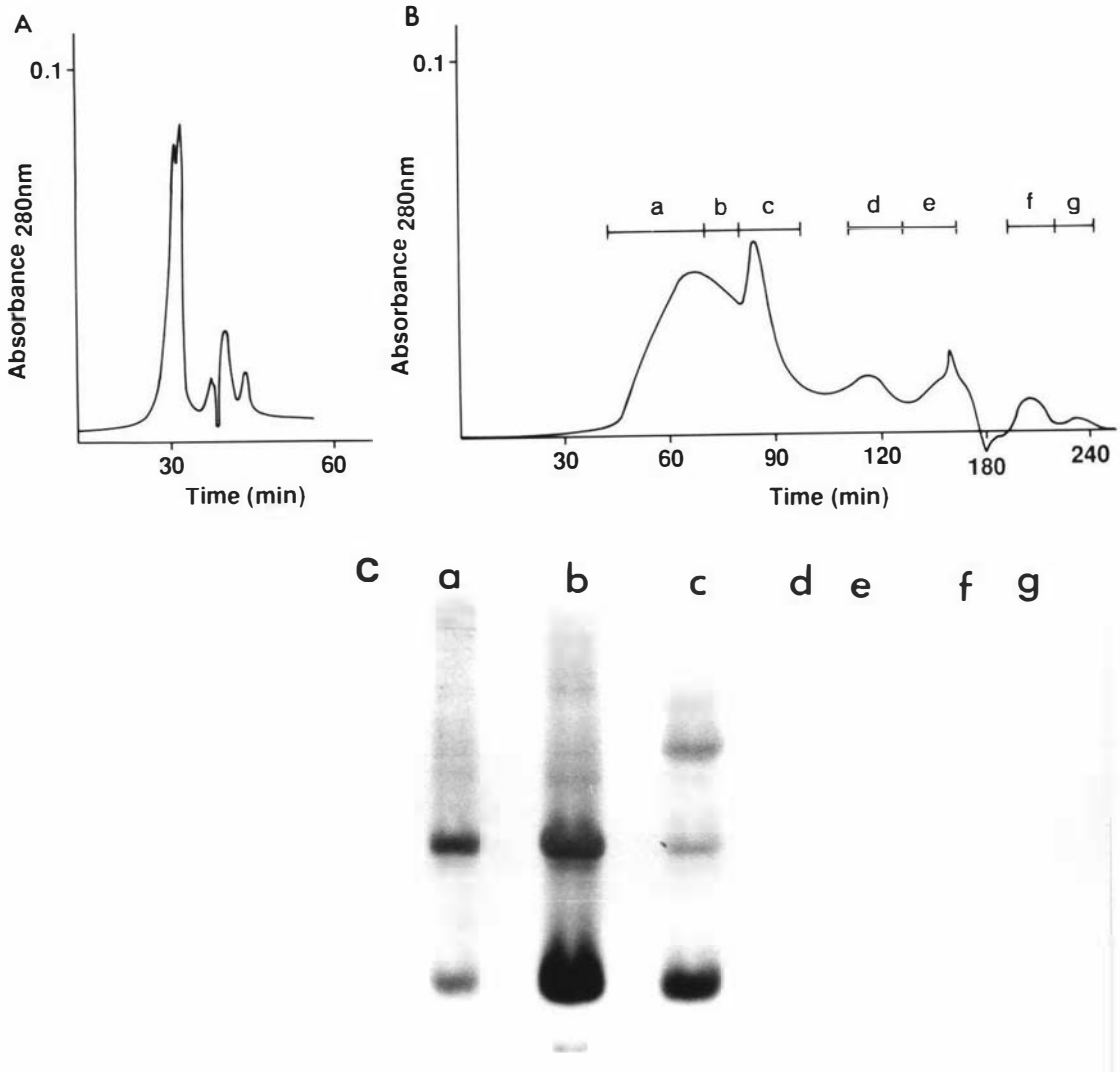

Fig.4.1 A,B,C HPLC profile of lipopigment proteins eluted from the TSK G2000 SW (A) and the TSK G2000 and G4000 SW columns connected in series (B). The columns were eluted with 0.1% LDS at a flow rate of 0.6 ml/min. The eluate was monitored at 280 nm. LDS-20% PAGE of pooled fractions a-g from the columns connected in series (C).

LDS-PAGE of the lipopigment proteins eluted from the size exclusion HPLC columns always showed the dominance of the 3.5 kDa protein band. However, as noted in the previous chapter, the amounts of the 14.8 and 24 kDa protein bands varied between lipopigment protein preparations and between gels of the same isolate (Fig. 4.2).

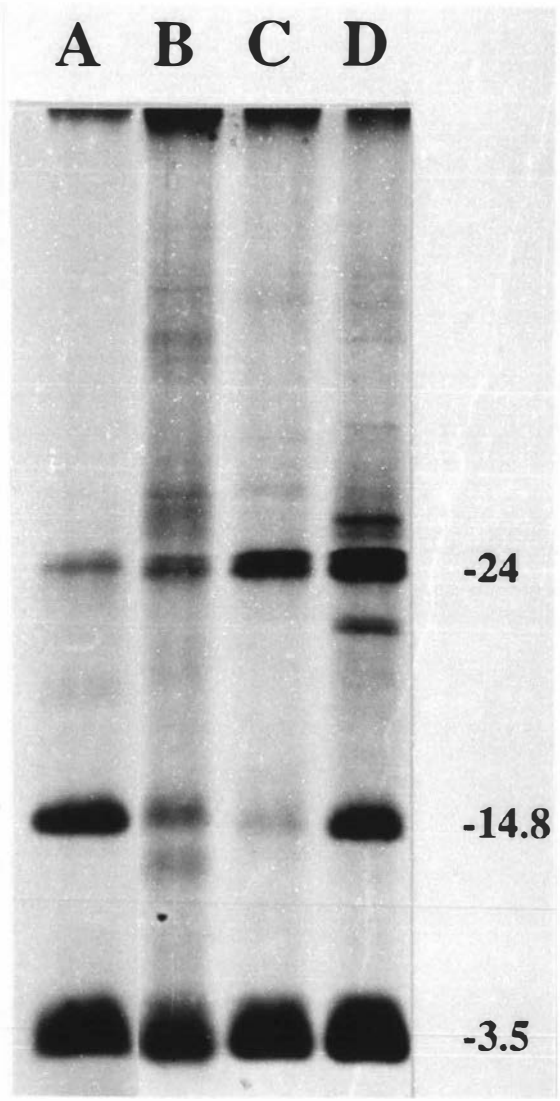

Fig.4.2 Silver stained LDS-20% PAGE of lipopigment proteins from pancreas (A), kidney (B), liver (C), and brain (D) after acetone precipitation and size exclusion HPLC (15 µg/lane). The numbers on the right hand side indicate apparent molecular weights calculated from the migration of molecular weight markers.

4.3.2 Amino acid sequencing of the molar dominant lipopigment protein

Because of the difficulty in separating the 3.5 kDa lipopigment band from the other components, fractions eluted from the HPLC containing all the lipopigment proteins were loaded onto the amino acid sequencer. This exploited the molar dominance of the 3.5 kDa protein indicated by silver staining of gels and ^{125}I labelling (Chapter 3). It was reasoned that should a clear sequence emerge, then a comparison of the initial sequence yield obtained with the total amount of lipopigment protein loaded should give an estimate of this dominant protein's contribution to the total lipopigment protein mass.

When total pancreatic lipopigment proteins after HPLC treatment were loaded onto the amino acid sequencer, a clear major sequence was obtained (Fig. 4.3).

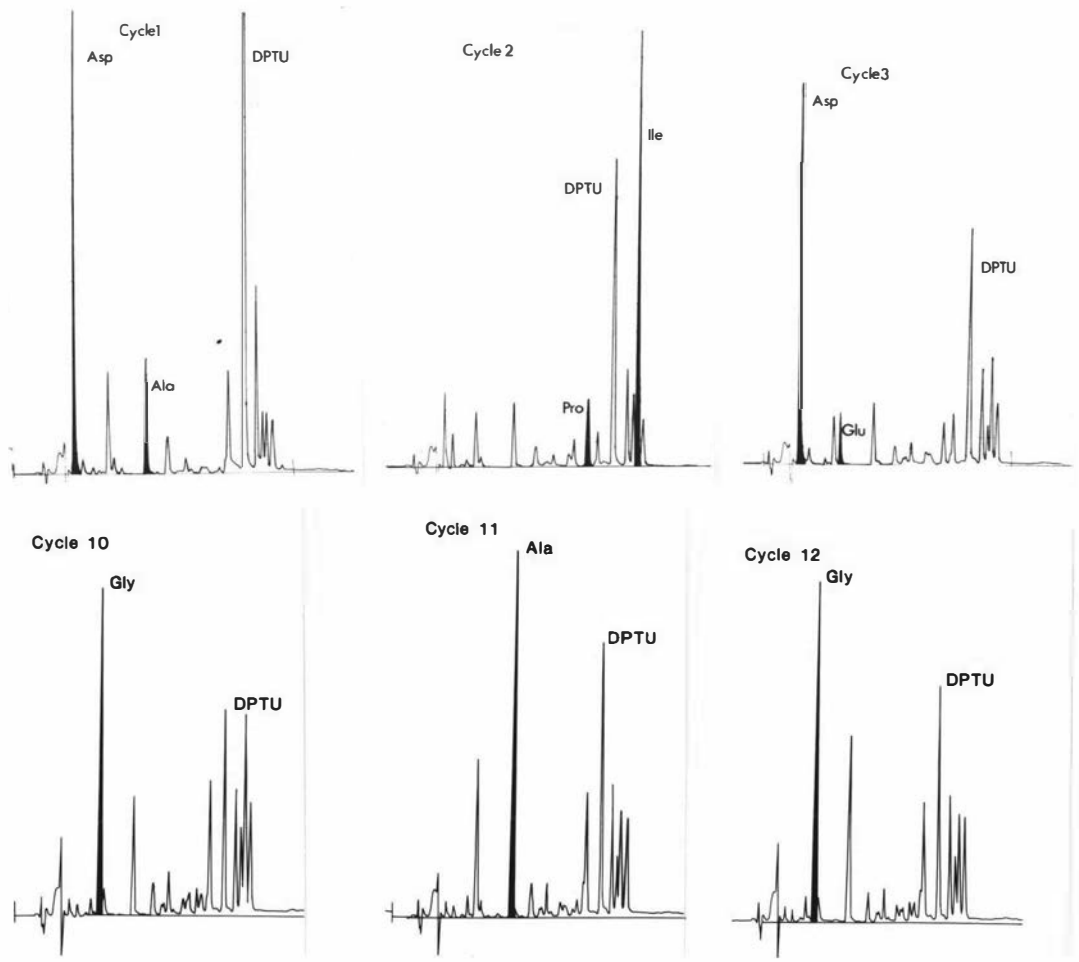

Fig.4.3 Cycles 1,2,3,10,11 and 12 obtained when pancreatic lipopigment protein containing 57.5×10^{-9} moles of hydrolysable amino acids was loaded onto the sequencer.

The sequence was determined to 40 residues in one experiment and confirmed to 24 and 20 residues in two other analyses. The same sequence was determined to 16 residues in kidney and to 18 residues in brain lipopigments. It was also determined to 14 residues in liver and kidney lipopigment proteins and to 17 in brain lipopigment proteins isolated from LDS solutions of total lipopigment by acetone precipitation alone, without subsequent size exclusion HPLC, with no significant difference in signal clarity.

The sequence found was as follows:

NH_2-Asp-Ile-Asp-Thr-Ala-Ala-Lys-Phe-Ile-Gly-Ala-Gly-Ala-Ala-Thr-Val-Gly-Val-Ala-Gly-Ser-Gly-Ala-Gly-Ile-Gly-Thr-Val-Phe-Gly-Ser-Leu-Ile-Ile-Gly-Tyr-Ala-Arg-Asn-Pro-.

This sequence is identical to the first 40 amino-terminal residues of the highly hydrophobic DCCD reactive proteolipid (also known as subunit *c*, the lipid binding subunit and subunit 9) of bovine and human mitochondrial ATP synthase (EC 3.6.1.34), a protein of 75 amino acids with a molecular weight of 7.608 kDa that is identical in both species (Sebald, *et al.*, 1979; Gay and Walker, 1985).

There was no sudden disjunction in the repetitive yields obtained for this peptide from any of the tissues examined, indicating that all the protein that initially coupled was at least 40 amino acids long, i.e. early cycles of the sequence did not contain amino acids arising from smaller amino-terminal fragments of the same peptide (Fig. 4.4).

The molar quantity of the sequenced peptide that initially coupled in the sequencer was calculated from the yields of the PTH amino acids (Fig. 4.4).

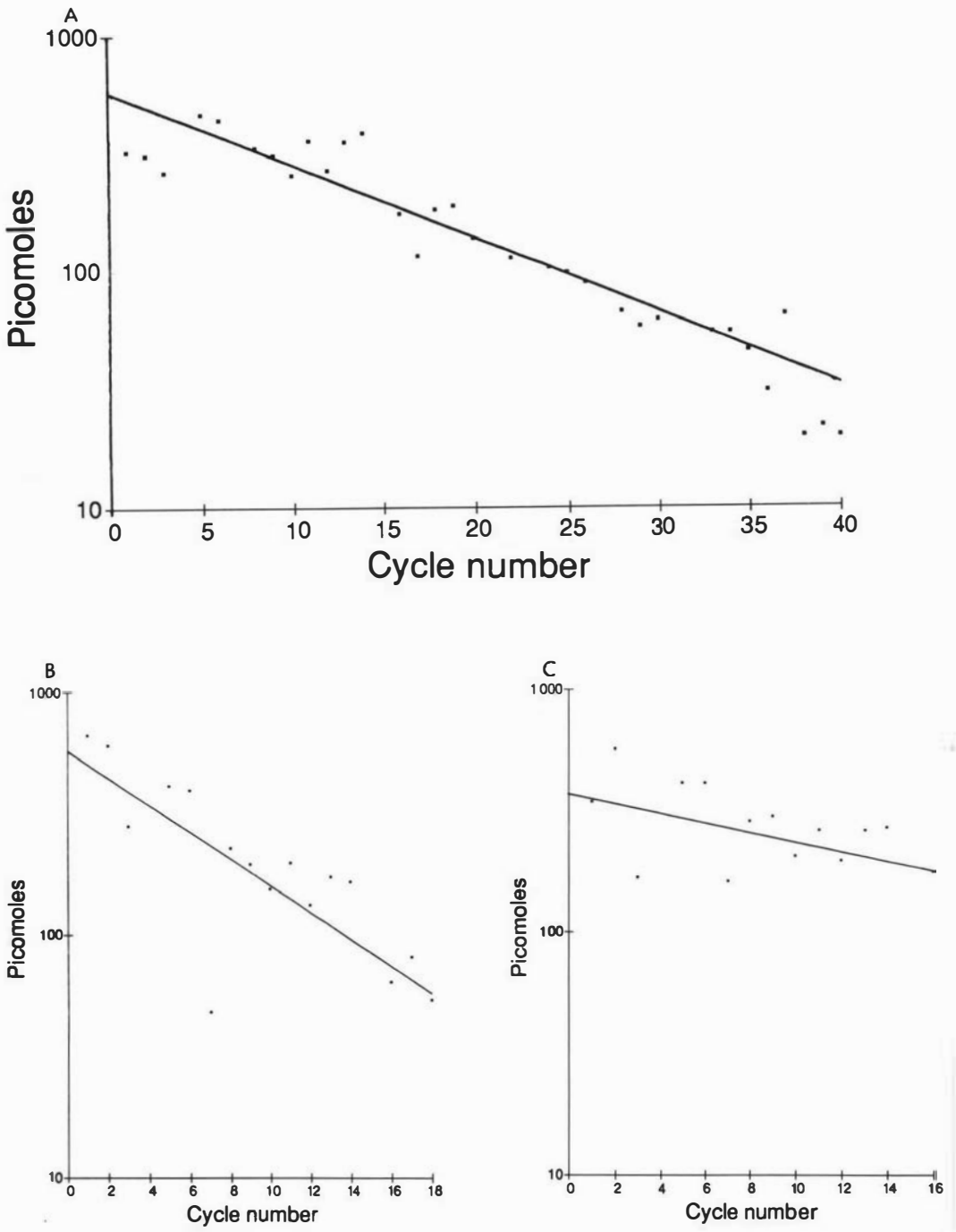

Fig.4.4 The PTH amino acid yields of the major sequence obtained from pancreas (A), brain (B), and kidney (C) lipopigment protein. Initial yields were read from the intercept of the repetitive yield slopes with the y-axis.

Estimates of the contribution of the peptide sequenced to the total lipopigment protein mass was made by comparing the initial yield of the sequenced peptide, with the total amount of lipopigment protein loaded onto the sequencer. For example, when pancreatic lipopigment proteins containing 57.5×10^{-9} moles of hydrolysable amino acids were loaded onto the sequencer, 0.579×10^{-9} moles of peptide coupled as the above sequence (Fig. 4.4). As this peptide is at least 40 amino acids long, it must contribute at least 40 x 0.579×10^{-9} moles of amino acids = 24.2×10^{-9} moles, to the total lipopigment protein mass. Therefore the sequenced peptide must account for at least $\frac{24.2 \times 10^{-9} \text{ moles}}{57.5 \times 10^{-9} \text{ moles}} \times 100 = 40.3\%$ of the total lipopigment protein.

Similar estimates for the contribution of the sequenced peptide to the total lipopigment protein mass from kidney and brain are shown (Table 4.1).

Table 4.1 Estimates of the contribution of the sequenced peptide to the total lipopigment protein masses

Source of lipopigment	Repetitive yield[a] (%)		Estimate of the sequenced peptide's contribution to the total lipopigment protein mass	
	No. of cycles		(%)[b]	(%)[c]
Pancreas[d]				
1.	40	92.8	40.3	75.6
2.	24	92.7	36.5	68.4
3.	20	ND[d]	40.7	76.3
Kidney[e]				
1.	16	94.3	41.0	76.9
2.	13	89.5	38.7	72.6
Brain				
1.	18	85.3	26.4	49.5

a. The repetitive yields were calculated from linear regression best fit plots of the logarithms of the yields at each cycle.
b. Calculated by multiplying the initial yield by 40 (the length of the sequenced peptide) and expressed as a percentage of the total hydrolysable amino acids loaded onto the sequencer.
c. Calculated by multiplying the initial yield by 75 (the length of the full subunit *c*).
d. Pancreatic lipopigment isolated from three affected sheep.
e. Kidney lipopigment isolated from two affected sheep.

A minor sequence was also determined in early cycles of pancreatic lipopigment protein analyses (Fig.4.3). It was as follows:

NH_2-Ala-Pro-Glu-Tyr-X-Ser-X-X-Ala-Met-Val- [X=unassignable].

This sequence was not clearly discernible in lipopigment protein from any other tissue due to high background levels.

Quantitative amino acid analysis of total lipopigment protein from pancreas, kidney and brain showed a high content of alanine, glycine, and leucine residues. This composition was similar to that of the full subunit *c* (Table 4.2).

Table 4.2 The amino acid composition, in moles % of total lipopigment protein and the full subunit *c* of mitochondrial ATP synthase.

	Total lipopigment			
Amino acid	Pancreas[a]	Kidney[b]	Liver[b]	Full subunit *c*[c]
Asp	5.3	5.6	5.2	4.0
Thr	6.2	6.1	5.1	4.0
Ser	5.3	6.0	6.0	6.7
Glu	5.1	4.9	6.0	4.0
Pro	2.7	2.0	4.1	1.3
Cys	0.5	ND[d]	ND	1.3
Gly	11.8	11.0	10.9	14.7
Ala	12.3	12.2	12.2	17.3
Val	8.3	7.4	5.7	5.3
Met	2.7	2.7	3.3	4.0
Ile	8.5	8.2	6.4	9.3
Leu	12.1	12.2	13.4	12.0
Tyr	4.3	5.1	3.6	2.7
Phe	7.9	8.2	8.4	9.3
His	1.2	1.1	1.5	0
Lys	3.0	3.5	4.1	2.7
Arg	2.8	3.8	4.1	1.3

a. Mean of 7 estimations
b. Mean of 4 estimations
c. Values taken from the cDNA sequence of the full ovine protein (Medd and Walker, unpublished)
d. Not determined

4.4 DISCUSSION

Lipopigment proteins were unable to be separated by size exclusion HPLC in LDS. Surprisingly, the lipopigment proteins with apparent molecular masses of 3.5, 14.8 and 24 kDa seen on PAGE eluted at size exclusion limits of up to 400 kDa (Fig.4.1). This was possibly due to the lipopigment proteins migrating in large dodecyl sulphate micellar aggregates. Difficulties in separating and purifying the lipopigment proteins led to the non-traditional approach of loading the proteins as a mixture onto the sequencer. The approach was based on the rationale that as the major low molecular weight 3.5 kDa protein was present in higher molar quantities than any other component, the major sequence obtained should therefore be of this protein. The sequence obtained was identical to the first 40 amino-terminal residues of a highly hydrophobic protein, subunit *c* of the mitochondrial ATP synthase. This protein's lipid-like solubility in neutral chloroform/methanol mixtures has led it to be classified as a proteolipid (Folch and Lees, 1951). This is an operational definition that does not imply the presence of covalently bound lipids.

The sequencing strategy not only allowed identification of the major lipopigment protein, but also enabled estimations to be made as to the contribution of this peptide to the total lipopigment protein mass. This was achieved by comparing the initial yield in the sequencer with the total amount of protein loaded. Values between 26-41% were obtained as estimates of the sequenced peptide to the total lipopigment protein masses from the amino-terminal sequences obtained (Table 4.1). These are minimum rather than actual values since the initial reactions of the Edman chemistry used in the sequencer are not quantitative and can vary between samples (Edman and Begg, 1967), and also because the length of the stored peptide remained to be established. The molecular weight of a 40 residue peptide is close to the 3.5 kDa demonstrated by LDS-PAGE. However, as similar proteolipids are known to give anomalous molecular weight estimations on dodecyl sulphate polyacrylamide gel electrophoresis (Farrell, *et al.*, 1988), the results obtained may not indicate the true molecular weight, or the length of the sequenced peptide. There are two possible explanations for such an anomaly. First a high detergent to protein

ratio in the migrating micelles, a consequence of the extreme hydrophobic nature of the protein making it bind more than the normal amount of detergent which results in a higher charge to mass ratio than normal (Lees, *et al.*, 1979). The second relates to incorrect assignment of the low molecular weight standards used to calibrate the apparent molecular weights of this protein (Kratzin, *et al.*, 1989).

Total lipopigment protein contained high contents of glycine, alanine and leucine (Table 4.2), consistent with a high proportion of the sequenced peptide being derived from subunit *c* of mitochondrial ATP synthase. The relative proportions of the amino acids did not allow any conclusions to be drawn as to the length of the stored peptide. The presence of histidine indicated that other minor proteins were also present, as histidine does not occur in subunit *c* of mitochondrial ATP synthase. No other mitochondrial ATP synthase components were detected in the sequence studies.

The minor sequence detected is related to the amino-terminal sequence of a 16 kDa protein isolated from mouse gap junction preparations (Walker, *et al.*, 1986) and to residues 7-17 of the proteolipid isolated from bovine vacuolar (chromaffin granule) ATPase (Walker, *et al.*, 1986; Mandel, *et al.*, 1988; Dermietzel, *et al.*, 1989). The significance of this minor sequence is not yet known. Its presence in lipopigment could be due either to minor contamination by membranes containing gap junctions or to the presence of a proteolipid fragment from a vacuolar ATPase.

Subunit *c* of mitochondrial ATP synthase has only previously been detected in mitochondria as part of the inner mitochondrial membrane F_o component of the oligomeric ATP synthase complex (Sebald and Hoppe, 1981; Walker, *et al.*, 1990). Lysosomes and other subcellular organelles also contain oligomeric proton pumping proteins with ATPase activity belonging to a class of vacuolar ATPases (Bowman, *et al.*, 1988a,b; Manolson, *et al.*, 1988; Zimniak, *et al.*, 1988). Lysosomal ATPase contains a subunit related, but not identical to the mitochondrial subunit *c* proteolipid (Moriyama and Nelson, 1989). The protein detected in lipopigment bodies is therefore not part of a lysosomal ATPase.

The full subunit *c* has properties similar to the specifically stored 3.5 KDa lipopigment protein. Both share the properties of poor Coomassie blue dye staining and aggregation behaviour on dodecylsulphate polyacrylamide gels (Palmer, *et al.*, 1986b; Fillingame, 1976; Graf and Sebald, 1978; Sebald and Hoppe, 1981). Physical properties described as being characteristic of the lipopigment stored in the ceroid-lipofuscinoses, are also similar to those of the full subunit *c*. These include a variable pH dependent chloroform/methanol extractability and similar thin layer chromatography migration behaviour to that of the so called "Schiff base polymer" that was thought to be formed as a result of lipid peroxidation (Sierra and Tzagoloff, 1973; Siakotos and Koppang, 1973). The complete extraction of lipopigments from Batten's disease in chloroform/methanol as well as the apparent extraction of autofluorescent material from histological preparations in acidified chloroform/ethanol has been documented (Srivastava, *et al.*, 1982). However, the lipid like behaviour of the proteolipid, poor Coomassie dye staining and a tendency to give inaccurate protein estimations by the Lowry method (Sebald and Hoppe, 1981) may help to explain why this type of protein was not recognised as being associated with these diseases in the past.

The data presented in this chapter does not indicate how long a fragment of the mitochondrial subunit *c* proteolipid is present in isolated lipopigment. If it is little more than 40 amino acids then it must account for at least 26-41% of the total lipopigment protein mass. If it is the full protein (7.608 kDa), then it accounts for 50-77% (Table 4.1), and some of the higher molecular weight lipopigment bands that are seen on LDS-PAGE are likely to be aggregates of subunit *c*. Aggregation in dodecylsulphate is a characteristic property of the full protein (Sebald and Hoppe, 1981), and could account for the anomalous LDS-PAGE and HPLC behaviour of the lipopigment proteins.

CHAPTER 5

THE CARBOXYL-TERMINAL DETERMINATION OF THE MAJOR STORED PROTEIN AND CHARACTERISATION OF THE OTHER LIPOPIGMENT PROTEINS

5.1 INTRODUCTION

When total lipopigment protein was loaded onto the sequencer, a clear sequence was identified. This sequence was shown to be identical with the first 40 amino-terminal residues of bovine and human subunit *c* of mitochondrial ATP synthase (Chapter 4). A minimum estimate of 40% was made for the contribution of the sequenced protein to the total pancreatic lipopigment protein mass on the basis that the full stored protein was at least 40 residues long. However, the sequencing studies described gave no indication that the carboxyl-terminal had been reached. Similarity between the physical properties of the full subunit *c* proteolipid, and lipopigment bodies stored in the ceroid-lipofuscinoses, suggested that the full protein might be stored. This chapter describes the carboxyl-terminal determination of the major stored protein and further characterisation of the 14.8 and 24 kDa lipopigment protein bands. Identification of subunit *c*, a proteolipid, as the major stored lipopigment protein component, enabled its further purification from lipopigment storage bodies by a specific extraction procedure.

5.2 SPECIAL MATERIALS AND METHODS

5.2.1 EXTRACTION OF PROTEOLIPIDS FROM LIPOPIGMENT

Isolated lipopigment bodies were solubilised by a modified Folch extraction (Folch, *et al.*, 1957). Lipopigment suspensions at 1-10 mg/ml were extracted with 20 vol of chloroform/methanol (2:1, v/v) containing 100 mM ammonium acetate, pH 7.2. After addition of 0.2 vol of deionized water the solution was gently shaken and left to stand at 4°C, until the aqueous and organic phases

separated. The proteins that precipitated at the interface were carefully withdrawn and retained. The lower organic phase was diluted with 1 vol of chloroform and the volume reduced to one fifth by rotary evaporation at 35°C. Proteolipids were precipitated from this extract with 10 vol of diethyl ether at -70°C for 1 h and harvested by centrifugation in a Sorvall GLC-1 centrifuge for 10 min at 1,400 g_{max} at 4°C. This proteolipid pellet and the proteins from the solvent interface were sequentially washed with methanol and deionized water and then dissolved in 1% LDS. At no stage were the proteins allowed to become dry.

5.2.2 DIFFUSION ELUTION FROM POLYACRYLAMIDE GELS

Pancreatic lipopigment containing 88 μg of protein was loaded into a 16-cm wide well in the stacking gel of a LDS-15% polyacrylamide gel. After electrophoresis, a side strip was cut from the gel and silver stained as described in sections 2.4-2.5. The bands containing the 3.5 and 14.8 kDa lipopigment proteins were cut from the gel, crushed in 10 ml of 1% LDS, and shaken for 18 h at 25°C. The supernatant was freeze dried, redissolved in 1 ml of deionized water and subjected to size exclusion HPLC as described earlier (section 4.2.1). The eluted protein fractions were collected and sequenced as described in section 4.2.2.

5.2.3 ELECTRO BLOTTING OF LIPOPIGMENT PROTEINS

Pancreatic lipopigment protein containing 10-20 μg of protein per lane was loaded onto LDS-15% polyacrylamide mini gels (1.5 x 8 x 10 mm).
Electrophoresis was carried out at 100 Volts for 2 h at 4°C, in a " Mighty Small Electrophoresis unit" (Hoefer Scientific Instruments). After electrophoresis the separated proteins were transfered onto polyvinylidene difluoride membranes (PVDF) according to the method of Matsudaira, (1987). The PVDF membranes were rinsed with 100% methanol and stored in the transfer buffer. LDS-15% polyacrylamide gels containing the separated lipopigment proteins were briefly soaked in transfer buffer, 10mM [cyclohexylamino]-1-propanesulfonic acid (CAPS), 10% methanol, pH 11.0. Electroelution was performed at 0.5 A in transfer buffer for 2 h at 4°C. After electroelution the PVDF membranes were washed in deionized water for 2-3

min, stained with 0.2% Coomassie blue R-250 in 50% methanol containing 10% acetic acid for 2-5 min, and then destained in 50% methanol containing 10% acetic acid for 5-10 min at 25°C. Coomassie blue stained protein bands of interest were cut into small pieces and placed in the cartridge block of a 470A gas phase sequencer, in the presence of only the Teflon seal, with neither polybrene nor glass fibre filter discs. The sequencing part of the experiment was performed by I.M. Fearnley and J. M. Skehel at the M.R.C. Laboratory of Molecular Biology, Hills Road, Cambridge, U.K.

5.2.4 CYANOGEN BROMIDE (CNBr) DIGESTION OF LIPOPIGMENT PROTEOLIPIDS

Lipopigment proteolipids, (0.5-1 mg) dissolved in chloroform/methanol (2/1, v/v) containing 100 mM ammonium acetate, pH 7.2, were precipitated with diethyl ether at -70°C, and then dissolved in distilled 98% formic acid (1.0 ml), containing CNBr (100 mg). This solution was gassed with nitrogen and left in the dark at 25°C for 24 h. Solvents were removed by drying *in vacuo* over NaOH pellets and the digest stored at -20°C until required.

5.2.5 MASS SPECTROSCOPY ANALYSIS OF CNBr DIGESTS

Mass spectra were obtained using a VG70-250S double focussing magnetic sector mass spectrometer (VG Analytical, Manchester, England) fitted with a VG liquid secondary mass spectrometry ion source and associated caesium ion gun. The CNBr digest of pancreatic lipopigment proteolipids were dissolved in distilled 98% formic acid. A 10 µl aliquot was suspended in a matrix of glycerol and loaded onto the stainless steel target of the sample insertion probe. Samples were bombarded with 35 keV caesium ions (ion current, 1-2 µA). The secondary ion beam was accelerated from the source at 8 keV and mass analysed at 5000 resolving power (RP). The magnet was scanned linearly between 890 and 1250 Da over 15 sec. Each analysis consisted of 4-6 scans of caesium iodide dissolved in water (reference ions, 912.3352 and 1172.1451) followed by 4-6 scans of the sample.

5.3 RESULTS

5.3.1 Characterisation of the proteolipids extracted from lipopigment

Lipopigments could be solubilised by a modified Folch extraction in chloroform/methanol (2:1, v/v) in the presence of 100 mM ammonium acetate. The solubility in these solvents was however variable between extractions. Amino acid analysis of the proteolipid fractions extracted from this solution by ether precipitation showed that almost 70% of the total protein present in lipopigment extracted was proteolipid (table 5.1).

Table 5.1 The proportion of protein recovered as proteolipid by diethyl ether precipitation of Folch extracted chloroform/methanol/ammonium acetate solubilised lipopigment bodies, expressed as moles % of the total amino acids present in lipopigment bodies.

Tissue	Proteolipid extracted (% of total protein)
Pancreas[a]	69.4±0.95
Liver[b]	69.9±1.77
Kidney[b]	68.7±1.60

a. Mean of seven estimations ± s.e.m.

b. Mean of four estimations ± s.e.m.

The protein component of the ether precipitated proteolipid and non-extractable material that precipitated at the interface during the Folch extractions was examined by LDS-PAGE. The major lipopigment protein bands with apparent molecular weights of 3.5, 14.8 and 24 kDa all extracted as proteolipids. In contrast, the nonextracted material that collected at the interface during the Folch extractions consisted of a number of minor, sharply focussed higher molecular weight components. The presence of the 24 kDa lipopigment band and traces of bands at 14.8 and 3.5 kDa indicated that the proteolipid extraction may not have been quantitative (Fig. 5.1).

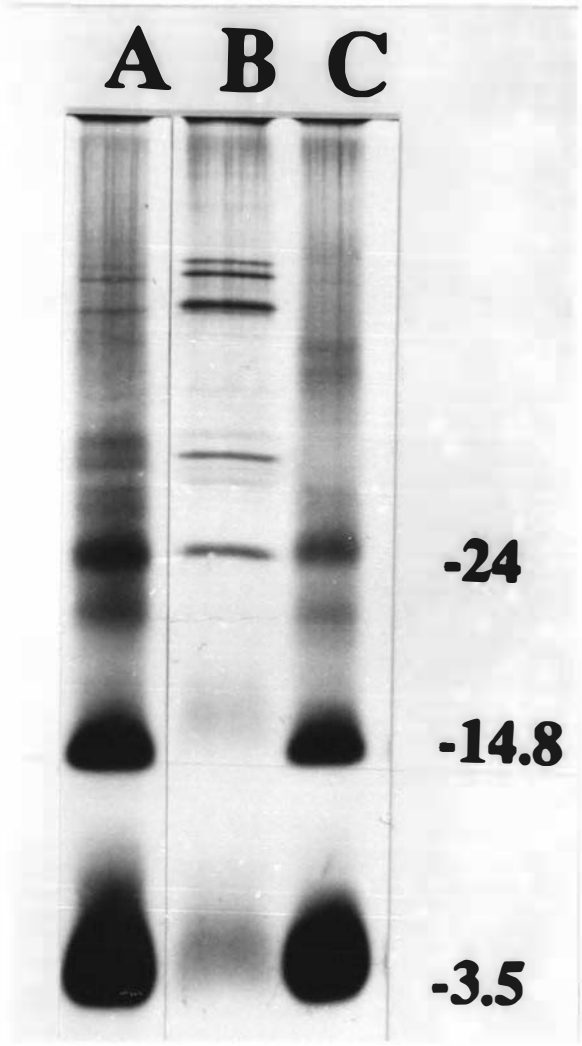

Fig.5.1 Silver stained LDS-20% PAGE of total pancreatic lipopigment proteins (A), nonextractable proteins (B), and extracted proteolipid fraction (C), The numbers on the right hand side indicate apparent molecular weights calculated from the migration of molecular weight markers.

The amino acid composition of the extracted proteolipid fractions containing high contents of glycine, alanine, and leucine residues, was consistent with the proteolipid fraction containing a significant portion of subunit *c* (Table 5.2). In contrast the nonextracted protein contained high contents of aspartic and glutamic acids, with low amounts of glycine and alanine residues. The 14.8 and 24 kDa protein bands seen on LDS-PAGE were either aggregates of subunit *c* or they were different proteins with similar amino acid compositions.

Table 5.2 The amino acid composition, in moles % of the nonextracted lipopigment protein, Folch extracted chloroform/methanol/ammonium acetate soluble lipopigment (proteolipid fraction),compared with that of the full subunit *c* of mitochondrial ATP synthase.

Amino acid	Nonextracted lipopigment			Extracted proteolipid			Full subunit *c*[c]
	Pancreas[a]	Kidney[b]	Liver[b]	Pancreas[a]	Kidney[b]	Liver[b]	
Asp	7.3	9.3	6.4	4.8	4.8	5.1	4.0
Thr	3.1	4.1	5.0	5.2	5.1	4.7	4.0
Ser	5.8	5.3	6.7	6.0	6.0	5.8	6.7
Glu	11.1	9.4	7.3	5.3	5.4	4.7	4.0
Pro	2.8	2.1	1.7	1.8	1.9	1.8	1.3
Cys	2.0	3.2	1.3	1.2	1.8	1.8	1.3
Gly	5.8	7.6	11.0	12.3	13.5	12.7	14.7
Ala	7.8	8.3	10.6	14.5	14.6	14.4	17.3
Val	8.3	7.6	8.4	8.3	7.1	7.3	5.3
Met	2.8	1.3	1.4	2.3	2.9	3.4	4.0
Ile	7.0	7.3	7.0	8.8	8.6	8.3	9.3
Leu	11.2	11.7	13.1	11.6	12.7	13.2	12.0
Tyr	2.8	2.0	6.2	3.8	2.8	3.1	2.7
Phe	4.8	4.3	8.5	7.7	7.7	8.1	9.3
His	3.4	4.1	0.6	0.7	0.9	0.8	o
Lys	7.5	5.3	3.1	3.1	2.6	3.0	2.7
Arg	6.5	7.1	1.5	2.6	1.6	1.8	1.3

a. Mean of seven estimations

b. Mean of four estimations

c. Values taken from the cDNA sequences of the full ovine protein (Medd and Walker, unpublished)

5.3.2 Identification of the 14.8 and 24 kDa lipopigment proteins

Purifying the 14.8 and 24 kDa proteins from the mixture of lipopigment proteins proved to be extremely difficult (chapter 4). When the 3.5 and 14.8 kDa Lipopigment proteins separated on LDS-PAGE were diffusion eluted from a gel and re-electrophoresed, the 14.8 kDa protein migrated as a distinct component, suggesting that it was a different protein, and not an aggregate of the major 3.5 kDa protein (Fig. 5.2).

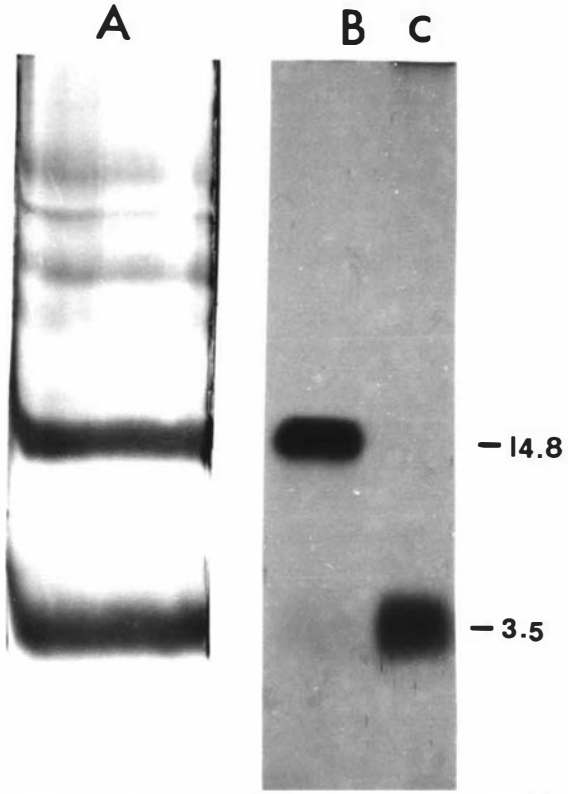

Fig.5.2 Silver stained LDS-15% PAGE of pancreatic lipopigment proteins, (A) and the 14.8 and 3.5 kDa lipopigment bands that were diffusion eluted from (A) and re-run on another LDS-15% polyacrylamide gel (B and C, respectively). The numbers on the right hand side indicate apparent molecular weights calculated from the migration of molecular weight markers.

However, amino acid sequencing of fractions that diffusion eluted from the 14.8 and 3.5 kDa lipopigment protein bands showed the first eight and four amino-terminal residues of subunit *c*, respectively. In order to further investigate this interesting result, lipopigment proteolipids separated by LDS-PAGE were electroblotted onto PVDF membranes so that direct sequencing of these bands could be investigated. Visualising the blotted bands was difficult as they stained poorly with Coomassie blue (Fig. 5.3).

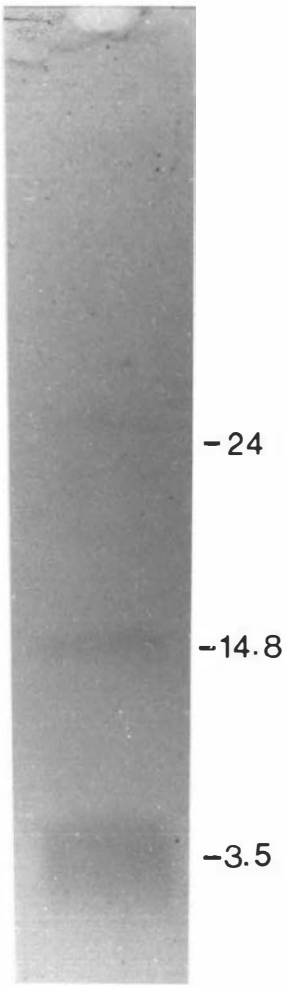

Fig.5.3

Coomassie blue stained pancreatic lipopigment proteolipids electroblotted onto a PVDF membrane from an LDS-15% polyacrylamide gel. The numbers on the right hand side indicate apparent molecular weights calculated from the migration of molecular weight markers. Note the poor Coomassie blue staining of the lipopigment proteolipids.

Sequencing of the bands blotted onto PVDF membranes confirmed the amino-terminal sequence residues 1-36 of subunit *c* from the 3.5 kDa lipopigment protein band. The same sequence was also obtained to 6 and 13 residues from the 14.8 and 24 kDa lipopigment bands. Initial yields of the 3.5, 14.8 and 24 kDa proteins were 21.8, 1.7 and 2.6 picomoles, respectively. These results indicated that the 14.8 and 24 kDa lipopigment protein bands were oligomers of the 3.5 kDa lipopigment protein. No other sequences were detected in these studies.

5.3.3 CNBr cleavage of lipopigment proteolipids and analysis of the digest fragments

In order to obtain sequence information beyond the amino-terminal 40 residues of the major stored lipopigment protein, CNBr digestion of the proteolipid extracted from pancreatic lipopigment was investigated. Products of the cyanogen bromide digestion were unable to be separated by reverse phase HPLC. Therefore an aliquot of the total digest was loaded onto the amino acid sequencer. Three sequences were assignable in the first few cycles. These were the expected amino-terminal sequence of subunit *c* and the sequences **Gly-Leu-Phe-** and **Val-Ala**, consistent with the

presence of Met^{60} and Met^{66} fragments of cyanogen bromide digestion of the full subunit *c* proteolipid. Further sequencing of these fragments was not possible due to the poor repetitive yields obtained. These small peptides were considered to be washed off the sequencer by the solvents used.

When the total CNBr digest products were subjected to mass spectral analysis two major peaks with masses of **976.5708** and **994.5897** Da in the range between **890** and **1250** Da were detected. These masses corresponded to those expected for the homoserine lactone and homoserine forms of the Carboxyl-terminal cyanogen bromide fragment of subunit *c*, (residues 67-75, Fig.5.5), calculated as **976.5871** and **994.5977** for $\mathbf{C_{51}H_{78}N_9O_{10}}$ and $\mathbf{C_{51}H_{80}N_9O_{11}}$, respectively (Fig. 5.4). Homoserine is formed from Met^{75} during the cyanogen bromide digestion. The other fragments expected from a CNBr digestion of the full subunit *c* were not detected.

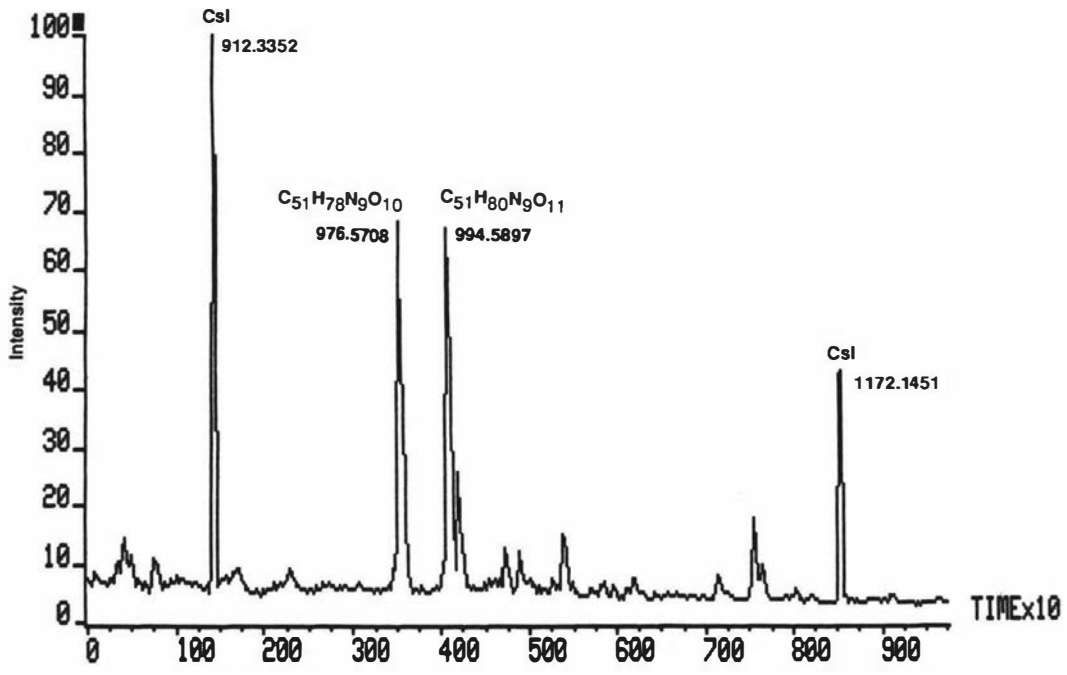

Fig.5.4 Linear mass spectral scan over the molecular ion region of the carboxyl-terminal CNBr cleavage fragment of subunit *c* extracted from pancreatic lipopigment. CsI calibrant signals were used for the mass measurement of the peptide fragments.

The presence of the carboxyl-terminal region of subunit *c* indicated that the full protein was stored in lipopigment but its complete normal structure could not be assumed until the complete sequence of the stored protein was determined (Fig. 5.5).

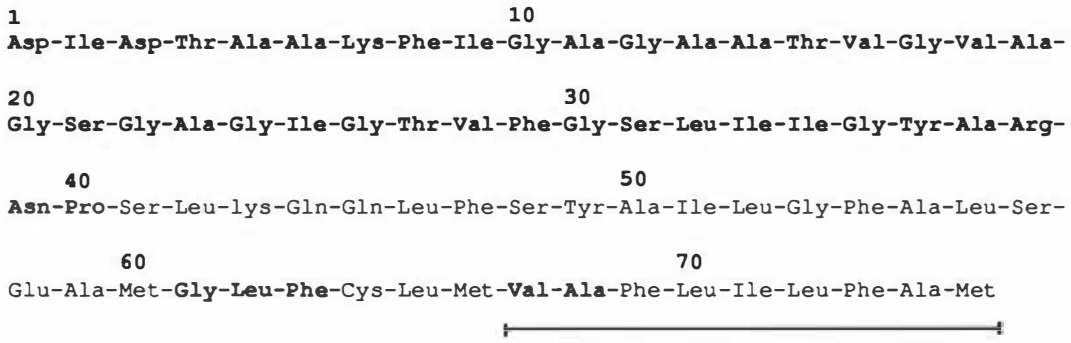

Fig. 5.5 Amino acid sequence of the major stored lipopigment protein. Residues identified by Edman degradation are shown in bold, those confirmed by mass spectroscopy of CNBr digestion are underlined.

5.4 DISCUSSION

There is a tendency for subunit *c* to form aggregates in dodecyl sulphate (Sebald and Hoppe, 1981). The variability of the 14.8 and 24 kDa bands on LDS-PAGE between different isolates of lipopigment and between different gels of the same isolate suggested that they might be aggregates of subunit *c* (Chapters 3 and 4). Identification of the amino-terminal sequence of subunit *c* as the only sequence detected from the 3.5 and 14.8 kDa lipopigment proteins that diffusion eluted from an LDS-polyacrylamide gel and from the 3.5, 14.8 and 24 kDa bands blotted onto PVDF membranes, showed that these bands were oligomers of subunit *c*. Isolated lipopigment protein was therefore composed largely of this protein. These findings explained the difficulties encountered in trying to separate and purify these proteins by conventional techniques using ion exchange chromatography on CM-23 Caboxymethyl Cellulose (Graf and Sebald, 1978) and LH-20 gel filtration chromatography (Cattell,*et al.*, 1971). It also strengthens the rationale used for the sequencing strategy described in Chapter 4, which was based on the major 3.5 kDa protein (subunit *c*) being present in higher molar quantities than any other protein. Although a number of different proteolipids are known to be present in whole mitochondria (Fearnley and

Walker, 1986), no other mitochondrial protein was detected in the lipopigment proteolipid fraction.

In conjunction with the present work, it has been established from cDNA studies that the normal ovine subunit *c* is identical to that of the bovine and human protein (Medd and Walker, unpublished). The full bovine and human subunit *c* proteolipids contain three methionine residues, at Met^{60}, Met^{66}, and at the carboxyl-terminus Met^{75}. If the full subunit *c* is present in ovine lipopigment then chemical cleavage with cyanogen bromide should give rise to three fragments. The amino-terminal fragment was not detected as it had a mass range beyond the capability of the instrument used. A fragment corresponding to cyanogen bromide cleavage at Met^{60}, was also not seen, possibly due to supression by other surface active components in the total digest or by the low surface activity of the fragment itself. The absence of expected species due to these effects when mixtures of digest products are subjected to mass spectral analysis is well documented (Barber, *et al.*, 1983; Biemann, 1982; Clench, *et al.*, 1985). The carboxyl-terminal cyanogen bromide fragment, which was identified by mass spectroscopy (Fig. 5.4), indicated that the full subunit *c* was present in lipopigment. This was also supported by the amino acid composition of the extracted lipopigment proteolipid fraction, which was very similar to that of the full subunit *c*. The contribution stored subunit *c* makes to total pancreatic lipopigment protein was therefore revised from a minimum of 40% to 73% (Table 4.1). This estimate is in accord with findings recorded in this chapter, i.e. that 70 % of the pancreatic lipopigment extracted as proteolipid. These values are both likely to be underestimates as the first is based on the initial yield in the amino-terminal sequencing which is not a quantitative reaction, and the second on proteolipid extraction which was not complete (Fig. 5.1).

The complete amino acid sequence of the stored protein was determined by collaboration with researchers at the M.R.C. Molecular Biology Laboratory, Cambridge, U.K. This was achieved after covalently attaching the products of the reactions of the lipopigment proteolipid fraction with cyanogen bromide and N-bromosuccinimide to 3-aminopropyl glass supports. The covalent coupling to solid supports was required to prevent the digest fragments from being washed off the sequencer in the solvents used (I.M. Fearnley, pers. comm.). These sequencing studies showed that previously unassigned residues 41 to 66 in the stored protein were identical to those of the full subunit *c* (Fig. 5.5).

Mass spectroscopy studies showed that there was no post-translational modifications to the carboxyl-terminus (Met^{66}-Met^{75}) nor was there evidence to indicate the presence of modified residues from both sequencing and amino acid analyses. This however does not preclude the possibility of the presence of acid labile modifications that would not have been detected by the studies described.

The subunit *c* proteolipid in the inner mitochondrial membrane is not known to be post-translationally modified (Sebald and Hoppe, 1981). There was no evidence for the presence in lipopigment fragments of subunit *c*, truncated at either amino or carboxyl terminals. It is concluded that the complete subunit *c* of mitochondrial ATP synthase is stored in ovine lipopigment.

The finding that the 14.8 and 24 kDa bands were oligomers of subunit *c* was also important as it precluded any other major protein component being specifically stored in the disease. As noted in chapter 4, this protein has only previously been detected in mitochondria as part of the F_0 component of the oligomeric ATP synthase complex, where it is estimated to account for 2-4% of the inner mitochondrial membrane protein (Sebald and Hoppe, 1981). As two thirds of the lipopigment mass is protein (Palmer, *et al.*, 1986b), subunit *c* alone accounts for at least half the total lipopigment mass. Its presence in lysosome derived lipopigment is therefore most likely to reflect the underlying biochemical lesion in ovine ceroid-lipofuscinosis.

CHAPTER 6
STUDIES ON MITOCHONDRIA ISOLATED FROM CONTROL AND AFFECTED SHEEP

6.1 INTRODUCTION

Lysosome derived ovine lipopigment bodies can be isolated relatively free of other contaminating cellular organelles or debris. The major stored lipopigment protein was identified as an inner mitochondrial membrane protein (subunit *c* of mitochondrial ATP synthase) in the previous chapters. It was necessary to exclude the possibility that the presence of subunit *c* could have arisen from contamination of lipopigment preparations with membranes of mitochondrial origin. The amounts of this protein in inner mitochondrial membrane preparations were also investigated to determine if accumulation of subunit *c* was occuring at the level of the mitochondrion. The functional integrity of mitochondria isolated from affected and control sheep were also compared by determination of their acceptor control ratios (ACR). This is defined experimentally as the ratio of respiration rate in the presence of added ADP (state 3) to the rate obtained following its depletion (state 4),(Fig.6.3). Efficiency of phosphorylation was determined as the ratio of the amount of ADP added to the amount of oxygen consumed during the burst of state 3 respiration. This experimental ratio equates with the theoretical P/O ratio i.e. mole equivalents of phosphate esterified per oxygen consumed. The P/O ratio gives values approaching 3 with NAD-linked substrates and 2 with succinate (Tzagoloff, 1982).

6.2 SPECIAL MATERIALS AND METHODS

6.2.1 ISOLATION OF MITOCHONDRIA AND INNER MITOCHONDRIAL MEMBRANE VESICLES FROM AFFECTED & CONTROL SHEEP

Mitochondria were prepared from affected and control liver and kidney tissues by the method of Nedergaard and Cannon (1979). Ovine liver and kidney tissues (10-20 g) were cut into small pieces, suspended in 4 vol of 0.28 M sucrose, 0.01 M Tris-HCl, pH 7.4 and homogenised with a Potter-Elvehjem homogeniser, firstly by making 10 passes using a motor driven pestle with a 0.6 mm clearance, followed by 10 passes with a pestle with a 0.3 mm clearance. Cell debris and nuclei were removed from the homogenates by centrifugation at 600 g_{max} for 5 min at 4°C in an SS34 rotor on a Sorvall RC-5 centrifuge. The supernatants were retained and

pelleted by centrifugation at 5,000 g_{max} for 15 min at 4°C. This pellet was dispersed in a solution of 0.3 M sucrose and repelleted by centrifugation at 7,500 g_{max} for 10 min at 4°C. Microsomal particles (the "fluffy layer") were washed off and the brown pellet resuspended in 0.3 M sucrose and sedimented again by centrifugation at 7,500 g_{max}. This procedure was repeated until a uniform brown pellet was obtained. The final pellet was suspended in 0.28 M sucrose, 0.01 M Tris-HCl, pH 7.4. Aliquots from these preparations were used for the respiratory measurements described below. Inner mitochondrial membrane vesicles were obtained by sonicating aliquots of the mitochondrial fractions (5-20 mg protein/ml), for 2 min in a Soniprep 150 sonicator at a 30 micron setting, followed by centrifugation at 7,500 g_{max} for 10 min. An aliquot was taken for thin section and negative staining electron microscopy. The rest of the inner mitochondrial preparations were dissolved in 1% LDS and stored at-20°C until required.

6.2.2 NEGATIVE STAINING ELECTRON MICROSCOPY

Inner mitochondrial membrane vesicles and isolated lipopigment bodies were negatively stained on 300 mesh formvar coated copper grids. Grids were floated on a solution of 1% (w/v) bovine serum albumin for 10 sec at 25°C. After removal of the excess bovine serum albumin by touching the grid with a piece of Whatman No 1 filter paper, they were transferred to sample suspensions containing isolated lipopigment and inner mitochondrial membranes, (0.4 mg of protein/ml) for 60 sec at 25°C. The grids were removed and washed with deionized water for 30 sec and then immersed in 1% potassium phosphotungstate, pH 7.2, for 10 sec. Excessive stain was removed by touching the grid with a piece of Whatman No 1 filter paper and the grid air dried at 25°C.

6.2.3 RESPIRATORY MEASUREMENTS

Respiration in the mitochondrial fractions was determined at 25°C by measuring the ADP stimulated oxygen uptake with a Clark type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.), by the method of Estabrook, (1967). The reaction mixture contained 1.6 ml of a buffered salt solution (45 mM potassium phosphate, 60 mM Tris-HCl, 5 mM $MgCl_2$, 2.5 mM potassium EDTA, pH 7.4) and 0.2 ml of 10 mM succinate or glutamate as respiratory substrate. After the addition of 990 µl of deionized water, the reaction mixture was saturated with air, and 0.2 ml of mitochondrial suspension (25-50 mg of protein/ml) was added, followed by

10 µl of 400 mM ADP a few minutes later to stimulate respiration.

6.3 RESULTS

6.3.1 Electron microscopy and LDS-PAGE of lipopigment and mitochondrial preparations

Isolated mitochondrial preparations were examined by thin section transmission electron microscopy using the same procedure described for checking purity of isolated lipopigment. Their ultrastructural appearance was quite distinct from that of the multilamellar profiles of isolated lipopigment bodies (Fig. 6.1 A,B). Negative staining electron microscopy of the inner mitochondrial membrane vesicles showed the stalked particles of the F_1 component of the mitochondrial ATP synthase complex on the outer surfaces of these inverted membranes (Fig. 6.1 D). These were not seen on the surfaces of the negatively stained isolated lipopigment bodies, which were relatively smooth in appearance (Fig. 6.1 C).

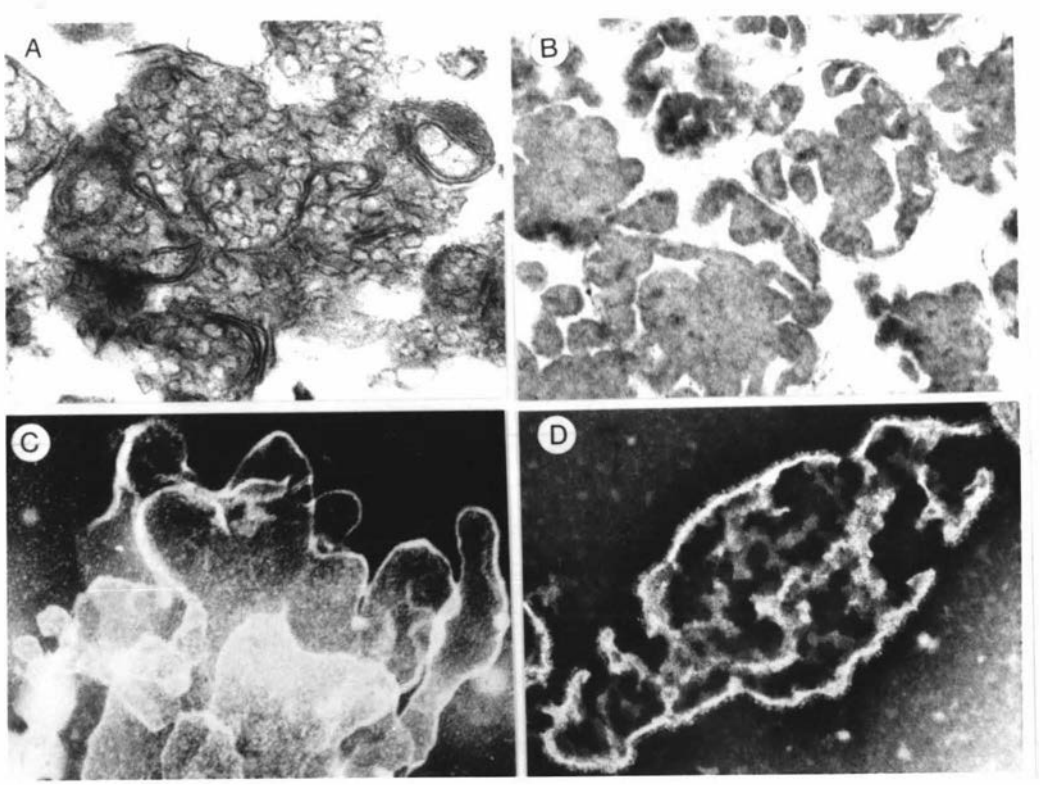

Fig. 6.1 Thin section electron micrographs of pancreatic lipopigment and isolated mitochondrial preparations (A and B,respectively) magnification x 46,000 and negatively stained pancreatic lipopigment and inner mitochondrial membrane vesicles (C and D,respectively) magnification x72,000.

LDS-PAGE of inner mitochondrial membrane vesicle isolates showed a large number of protein bands. The presence of subunit *c* in the region where it is known to migrate on LDS polyacrylamide gels (with an apparent molecular weight of 3.5 kDa) was only barely visible in preparations from both control and affected sheep despite the use of the silver stain developed to visualise this protein in lipopigments, where it stains as a dominant band at 3.5 kDa (Fig. 6.2 A). PAGE of purified bovine ATP synthase showing the presence of subunit *c* and other components of the complex is presented for comparison courtesy of I.M.Fearnley, R.Lutter and J.E.Walker (Fig. 6.2 B).

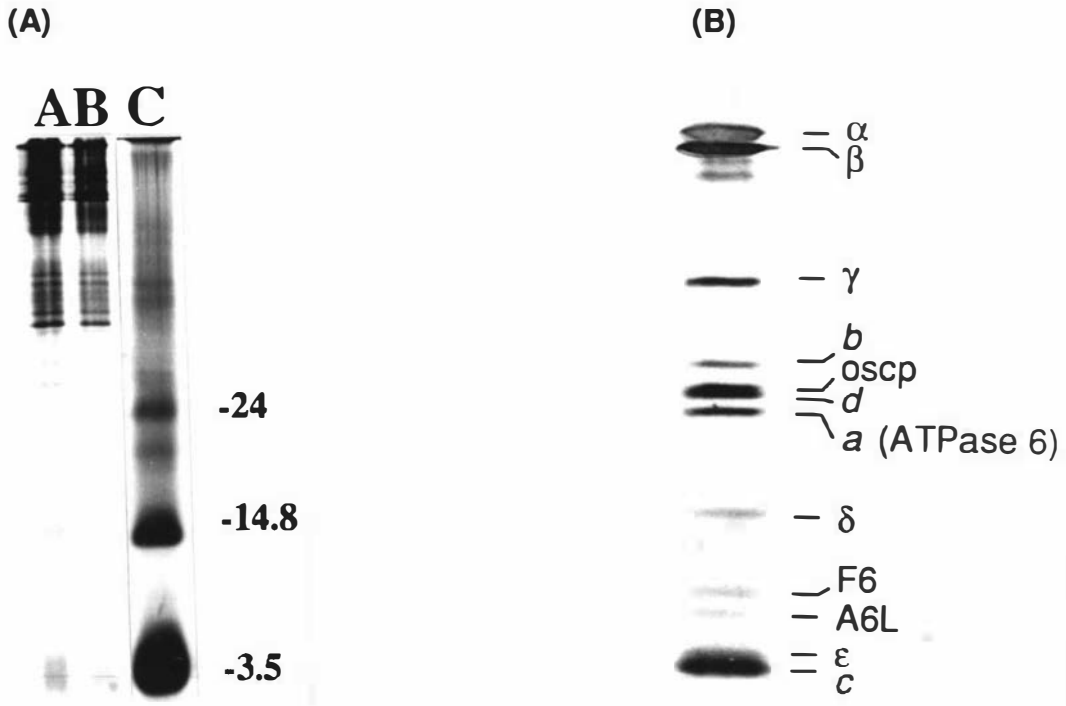

Fig.6.2 Silver stained LDS-PAGE of (A) inner mitochondrial membrane vesicle proteins from control and affected sheep livers (lanes A and B, respectively, 20 µg/lane) and pancreatic lipopigment proteins (lane C,15µg/lane). (B) PAGE of purified bovine ATP synthase courtesy of I.M.Fearnley, R.Lutter and J.E.Walker.

6.3.2 Respiratory measurements on isolated mitochondrial fractions

Respiration rates and acceptor control ratios of isolated mitochondrial fractions from affected and control sheep kidney tissue were determined by measuring oxygen uptake with the Clark type oxygen electrode (Fig. 6.3).

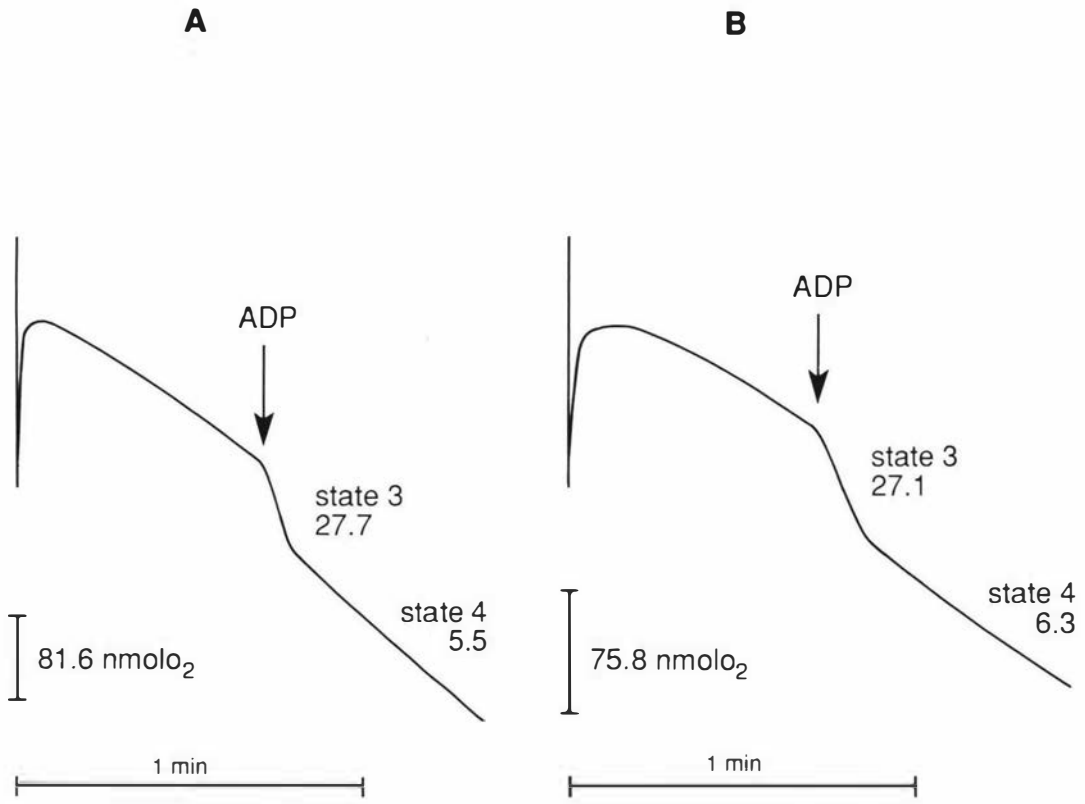

Fig.6.3 Oxygen traces obtained when the respiratory activity of isolated mitochondrial preparations from affected (A) and control (B) kidney tissues were assayed by the procedure described in section 6.2.3. Glutamate (10 mM) was used as respiratory substrate and stimulation of State 3 respiration was by the addition of 400 mM ADP. The numbers for State 3 and 4 on the traces are oxygen uptakes expressed as nmol min^{-1} mg $protein^{-1}$.

Stimulation of respiration (state 3) was observed in both affected and normal mitochondrial preparations after the addition of ADP. After exhaustion of ADP the rate of respiration decreased (state 4). Calculated acceptor control ratios (i.e. the rate of oxygen consumption in the presence of ADP compared with the rate after ADP had been converted into ATP) of between 2.3-5.0 were obtained from both affected and control mitochondrial preparations (Table 6.1). Control and affected kidney mitochondria oxidised 10 mM succinate at rates of 3.2 and 3.4 μmoles of O_2 h^{-1} mg of protein $^{-1}$ respectively. These rates were similar to those reported in the literature for sheep liver mitochondria (Reid and Husbands, 1985).

Table 6.1 Respiratory control ratios from control and affected kidney mitochondrial preparations using succinate and glutamate as substrates

Oxygen uptake (nmoles min^{-1} mg $protein^{-1}$)				ACR (state 3/ state 4)	
succinate		glutamate		succinate	glutamate
state 3	state 4	state 3	state 4		
Control[a]					
1. 60.4	19.8	27.1	6.3	3.0	4.3
2. 45.1	19.9	38.2	12.2	2.3	3.1
Affected[b]					
1. 54.8	17.2	27.7	5.5	3.2	5.0
2. 26.9	18.5	24.2	6.4	3.1	3.8

a.Kidney mitochondrial preparations isolated from two control sheep
b.Kidney mitochondrial preparations isolated from two affected sheep

The measured ADP/O ratios from both affected and control mitochondrial preparations were close to those theoretically expected for isolated coupled sheep mitochondria (Table 6.2).

Table 6.2 ADP/O ratios from control and affected kidney mitochondrial preparations using succinate and glutamate as substrates

	Oxygen uptake when 400mM ADP was added (nmoles)		ADP/O ratios	
	succinate	glutamate	succinate (2)[a]	glutamate (3)[a]
Control[b]				
1.	111.1	75.8	1.8	2.6
2.	140.4	83.2	1.4	2.4
Affected[c]				
1.	117.3	81.6	1.7	2.5
2.	111.1	80.8	1.8	2.5

a. Expected theoretical ratio

b. Kidney mitochondrial preparations isolated from two control sheep

c. Kidney mitochondrial preparations isolated from two affected sheep

6.4 DISCUSSION

The quantitative data presented in Chapter 4 and 5 showed that subunit *c* was stored in lipopigment isolated from sheep with ceroid-lipofuscinosis. Evidence that it could not have arisen from contamination of lipopigment preparations with inner mitochondrial membranes was obtained by a combination of electron microscopy and polyacrylamide gel electrophoresis. Firstly, despite the common use of sonication and centrifugation steps in the methods of isolation of lipopigment and mitochondrial preparations, they had quite separate and distinctive ultrastructural appearances. In negatively stained preparations there was no indication of the stalked particles of the F_1 component of the ATP synthase complex on the surfaces of lipopigment bodies, whereas these were clearly seen on the inner mitochondrial vesicles (Fig. 6.2 C & D). Secondly, the protein composition of inner mitochondrial vesicles seen by LDS-PAGE was distinct from that of isolated lipopigment, with subunit *c* being barely visible in the former. Therefore any contamination of lipopigment bodies by mitochondrial membranes would tend to diminish the concentration of subunit *c* in the lipopigment, rather than be a source of it. Further support for a lack of mitochondrial contamination of isolated lipopigment bodies comes from the lipid analysis of Palmer, *et al.*, (1986a), which showed that lipopigment lipids were those characteristic of lysosomal membranes with no indication of the presence of the distinctive mitochondrial phospholipid, cardiolipin.

The quantitative data presented in Chapters 4 & 5 and observations recorded and discussed above, indicate that subunit *c* of mitochondrial ATP synthase is specifically stored in lipopigment from sheep with ceroid-lipofuscinosis and its presence was not due to mitochondrial contamination of isolates of lipopigments. Similar amounts of subunit *c* in inner mitochondrial membranes of affected and control preparations (Fig. 6.2 A) indicated that accumulation of this protein in the disease was not occuring in the mitochondrion.

Acceptor control ratios of mitochondrial preparations from affected sheep kidney tissue were comparable to those determined from normal sheep kidney tissue. They were also consistent with those reported in the literature for sheep liver mitochondrial preparations (Reid and Husbands, 1985).
Measured respiration rates and P/O ratios were close to those theoretically

expected using succinate or glutamate as substrates. This indicated that the mitochondrial fractions from affected sheep maintained a structural and functional integrity enabling acceptor control comparable to those from control mitochondrial fractions. The lesion in the disease was therefore unlikely to be related to a mitochondrial respiratory chain or an oxidative phosphorylation defect related to the mitochondrial ATP synthase complex.

CHAPTER 7

CELL CULTURE OF OVINE KIDNEY EPITHELIAL CELLS

7.1 INTRODUCTION

The recognition of subunit *c* of mitochondrial ATP synthase as the major stored component in isolated lipopigment bodies from the ovine form of ceroid-lipofuscinosis (Chapters 4,5,6) suggests that its storage reflects the primary lesion in the disease. In order to further study the pathogenesis of subunit *c* storage the development of an *in vitro* cell culture model of the disease was investigated using cultures of kidney cells. The need to obtain a radiolabelled subunit *c* as a potential substrate was envisaged and sheep as an animal were too large for this to be achieved *in vivo*. Kidney tissue was chosen because it is rich in mitochondria, has relatively small amounts of connective tissue and in lambs affected with ceroid-lipofuscinosis there is storage of lipopigment. Although biochemical studies on fibroblast cell lines from fibroblasts derived from patients with various forms of ceroid-lipofuscinoses have been reported in the literature (Dawson and Glaser, 1987; 1988), they give no indication of the presence of ultrastructural lesions characteristic of lipopigment storage bodies. This chapter describes the culturing of kidney cell monolayers from affected and control sheep. The primary culture was investigated initially as it was considered to most closely resemble the *in vivo* situation. However, logistics and number of animals available dictated that cells passaged several times also be used. Initial experiments were designed to determine if epithelial cell cultures could be obtained and maintained at confluence and to investigate the occurrence of lipopigment bodies in cultured cells.

7.2 SPECIAL MATERIALS AND METHODS

7.2.1 COMPOSITION OF GROWTH AND MAINTENANCE MEDIA

The growth medium used in cell culture experiments consisted of Eagles minimum essential media (MEM) with 1.5% $NaHCO_3$, pH 7.4, suplemented with 10% (w/v) tryptose broth, 10% (v/v) foetal bovine serum (FBS), 2% MEM vitamin solution and a 1% (v/v) solution of antibiotics (PSK) containing penicillin (100 units/ml), streptomycin (10 mg/ml) and kanamycin (10 mg/ml). In some experiments the 1% PSK solution was omitted.
The maintenance media consisted of MEM suplemented with 2% FBS, 2% MEM vitamin solution and a 1% solution of PSK.

7.2.2 PREPARATION OF AFFECTED AND CONTROL KIDNEY EPITHELIAL CELLS FOR PRIMARY CELL CULTURE

Kidneys from affected and control sheep were removed under sterile conditions within minutes of euthanasia. The cortical layer was removed, minced finely with scalpel blades and rinsed with 100 ml of 0.01 M phosphate buffered saline, pH 7.4, until a clear supernatant was obtained. The cell suspension was transferred to a sterile 250 ml conical flask and 25 ml of 0.3% (w/v) trypsin (1:250 Difco Certified) added and allowed to incubate at 37°C for 10 min. The supernatant was then discarded and a further 25 ml of 0.3% (w/v) trypsin added. The incubation was continued a further 30 min, the cell suspension filtered through a fine wire-mesh filter and 10 ml of 10% FBS added to the filtrate to neutralize the effect of the trypsin. Cells were harvested by centrifugation at 200 g_{max} in a Sorvall GLC-1 centrifuge for 10 min and resuspended in 20 ml of growth medium. A 200 µl aliquot of the cell suspension was mixed with 1.8 ml of trypan blue 0.2% (w/v) and the transparent live cells counted.

7.2.3 GROWTH AND MAINTENANCE OF CELL CULTURES

Cells were distributed at 1 x 10^6 cells/ml into 25cm^2 plastic culture flasks and in Leighton tubes containing glass cover-slips.

They were placed in an incubator at 37°C and left undisturbed to form monolayers. At confluence, the monolayer cells were washed with PBS and harvested by treatment with 2 ml of antibiotic-trypsin-versene (ATV) mixture containing penicillin (100 units/ ml), streptomycin (10 mg/ ml), trypsin 5% (w/v) and versene (EDTA, tetrasodium salt, 2% (w/v)) for 5 min and resuspended in 10 ml of growth medium. After counting the cells they were passaged at 1 x 10^6 cells/ml into fresh culture flasks and incubated at 37°C to form monolayers as before. Cells at confluence in some flasks were maintained in maintenance media with changes to fresh media every 48 h until the monolayers started to degenerate and peel off the culture flasks at senescence.

7.2.4 PREPARATION OF CELLS FOR LIGHT AND THIN SECTION ELECTRON MICROSCOPY

Monolayer cells in 25 cm^2 culture flasks were washed in PBS and then fixed in 2 ml of 3% glutaraldehyde in 0.01 M phosphate buffer at pH 7.2 for 20 min. Fixed cells were gently scraped from the flask with a thin metal spatula, collected by centrifugation and processed for electron microscopy as described in section 2.3. On reaching confluence, cells in Leighton tubes were washed with PBS and stained by haematoxylin and eosin (H&E), and by luxol fast blue.

7.2.5 RADIOLABELLING OF CULTURED CELLS

Confluent monolayers in 25cm^2 flasks, were washed with PBS and placed in PBS for 4 h at 37°C. They were then incubated with 10 µl of a ^{3}H-amino acid mixture (1.0 mCi/ml) in PBS containing 2% FBS, and 1% PSK antibiotics. After 30, 60, 120 and 360 min incubation, the labelled amino acids were removed and cells washed with PBS. Some flasks were treated with 2 ml of ATV, for 2-5 min, resuspended in 10 vol of PBS and harvested by centrifugation at 200 g_{max}. Others were placed in 5 ml of maintenance media and incubated for a further 24 h prior to ATV treatment and harvesting as above. The cell pellets were resuspended in 1 ml of deionized water. A 100 µl aliquot was used for protein determination by the method of Lowry, *et al.*, (1951).

The radioactivity in the cell protein was measured by treating a 10 µl aliquot of the cell pellet with 1 ml of 10% trichloroacetic acid (w/v) and 0.5 ml of bovine serum albumin (1 mg/ml) at 4°C. After centrifugation, the supernatant was discarded and the precipitate dissolved in 500 µl of 0.6 N NCS tissue solubiliser and added to 2.5 ml of scintillant solvent (Triton X-100/toluene 1:2 (v/v) containing 0.4 % 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP)). The radioactivity was measured by liquid scintillation counting on a Beckman LS 7500 or a Beckman LS 8000 Series Liquid Scintillation Spectrometer. Counting efficiencies of the order of 25% for ^{3}H were determined by the H-number method and the radioactivity expressed as disintegrations per minute (dpm). Incorporation of ^{3}H into cellular proteins was determined by the dpm recovered in the TCA precipitable fractions compared to total dpm present. The remainder of the harvested cell pellet was dissolved in 10 vol of chloroform/methanol (2:1,v/v) containing 100 mM ammonium acetate and divided into two equal aliquots. After the addition of 0.2 vol of lipopigment dissolved in the same solvent to one aliquot, the proteolipid fractions were extracted by the procedure described in section 6.2.1, dissolved in 1% LDS and stored at -20°C until required. The amount of radioactivity in the cell proteolipid fractions was measured as above. The radioactivity amongst the proteolipid proteins separated by LDS-PAGE was determined by cutting the gel into strips, incubating them in a 5 ml solution of 99 parts of a 33% hydrogen peroxide solution containing 1 part ammonia at 60°C for 24 h. After leaving the solutions to cool to room temperature, 0.5 ml aliquots were added to scintillation solvent, left in the dark for a further 24 h and counted as above.

7.3 RESULTS

7.3.1 Morphology and growth characteristics of cultured kidney cells

Primary cell culture monolayers were obtained from both control and affected kidney epithelial cells under the described growth conditions. They reached confluence between 24-48 h and were able to be maintained at confluence for up to 6 days, before reaching a senescent stage, when the monolayers started to peel off the culture flasks. Cultured epithelial cells retained their characteristic closely packed polygonal morphology up to about the 5th passage when spindle shaped fibroblast-like cells became more abundant. The primary cell morphology was retained in early passage cells (Fig. 7.1).

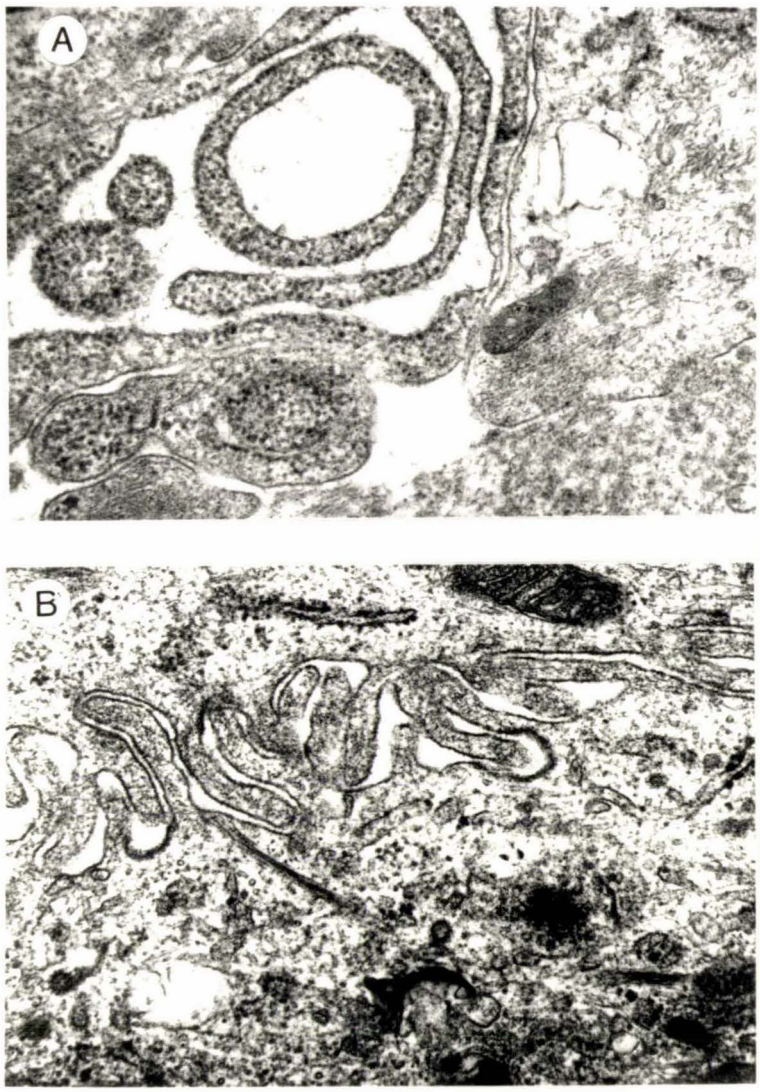

Fig.7.1 Thin section electron micrographs of affected kidney epithelial cell in primary culture (A), and in third passage culture (B), magnification x 31,800.

Similar growth rates were shown by both control and affected primary cell cultures (Fig. 7.2).

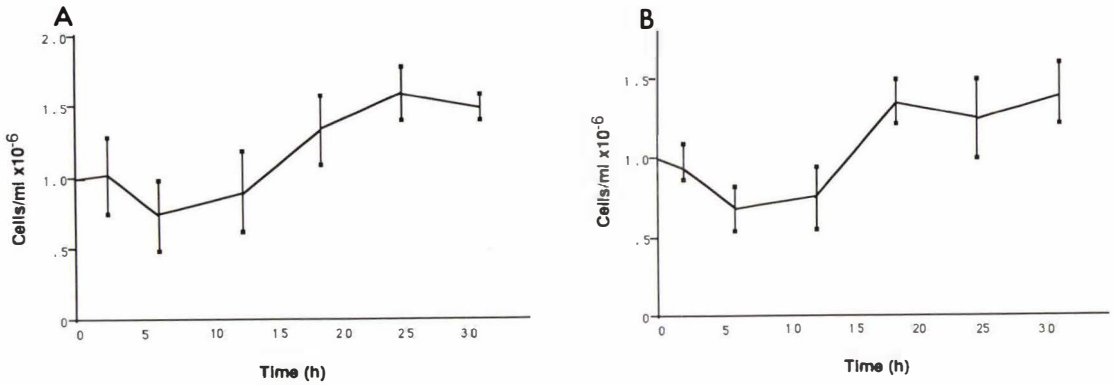

Fig. 7.2 The growth of affected (A) and control (B) kidney epithelial primary cultures that were distributed at $1x10^6$ cells/ml. Points on the graphs represent cells harvested from small (25 cm^2) culture flasks, and counted by the procedure described in section 7.2.2.

Affected and control cultured cells also had similar appearances when stained with H & E and luxol fast blue stains (Fig. 7.3). The latter stain failed to show the presence of lipopigment bodies in affected cultured cells.

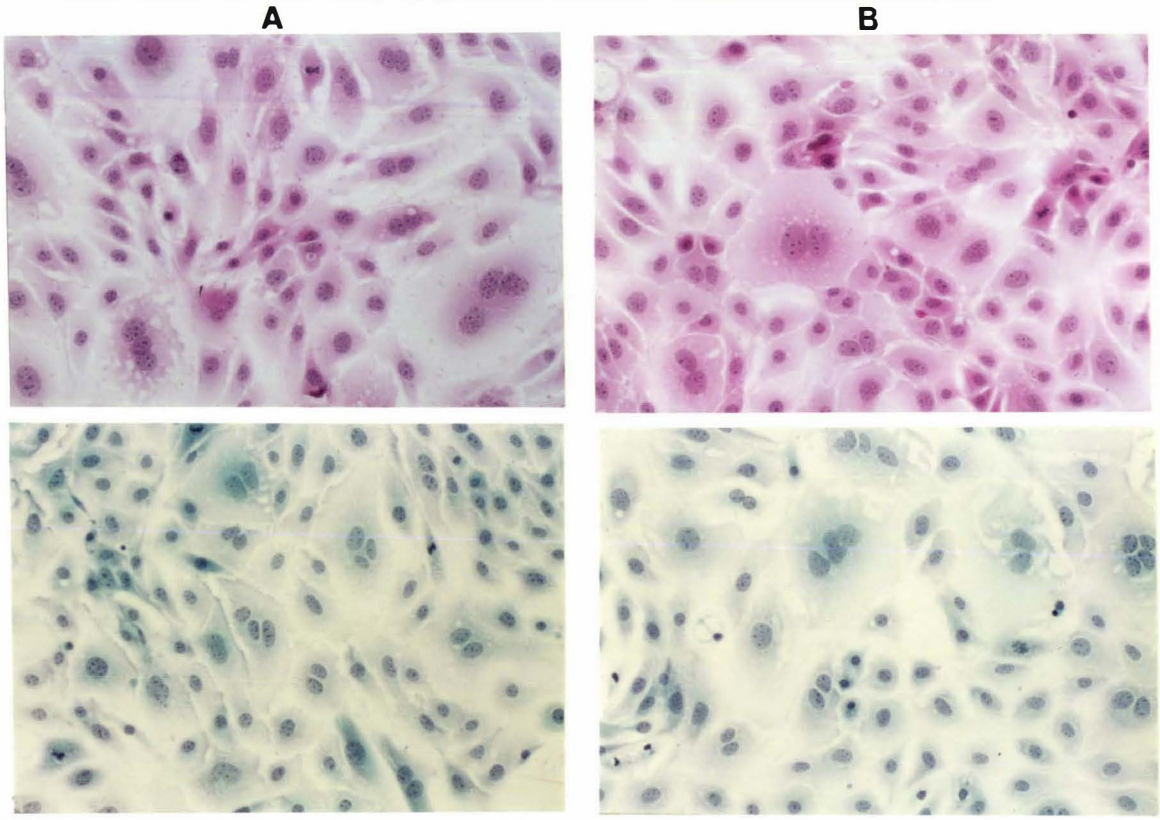

Fig. 7.3 Light microscopic appearance of affected (A) and control (B) primary cultured cells stained with H&E and luxol fast blue. Magnification x 25.

Membrane bound cytoplasmic bodies containing multilamellar and pentalaminar structures were sometimes observed in thin section electron micrographs of affected primary cell cultures and in early passaged cells reaching confluence (Fig. 7.4 A,B). These structures were observed in the presence or absence of antibiotics in the growth media and were not seen in control cells under either circumstance. They were present in approximately 1 cell out of 100 examined and appeared similar to those of lipopigment storage bodies seen *in situ* in affected tissue. Heterogeneous collections of autophagic structures in control (Fig. 7.4 C,D) and affected cells were also observed more frequently.

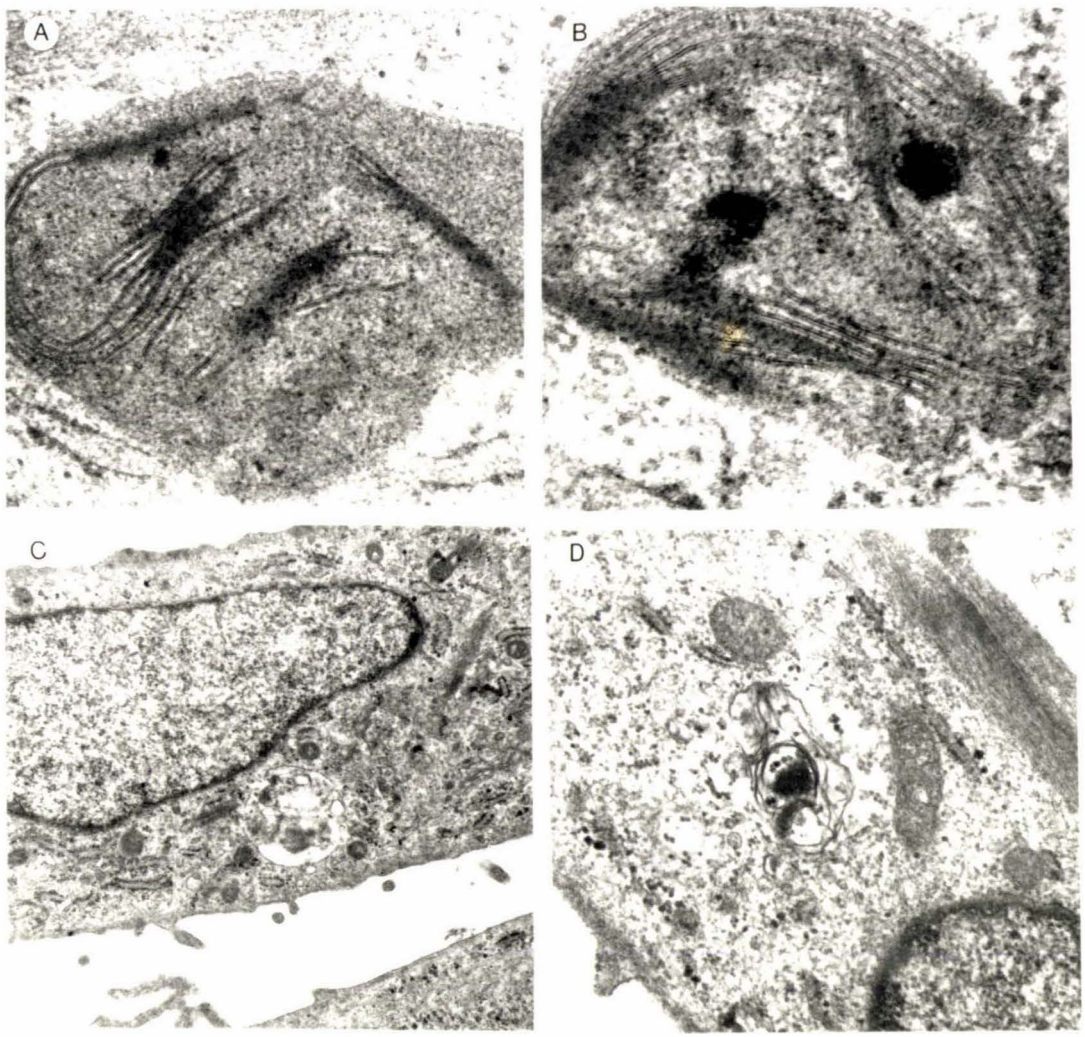

Fig. 7.4 Thin section electron micrographs of membrane bound cytoplasmic bodies from affected cultured cells (A & B), magnification x 72,000 and autophagic structures from control cells (C & D), magnification x 15,300 & 31,800 respectively.

Delineation of structures resembling those of lipopigment bodies from cell cultures that were maintained at confluence to senescent stages was difficult due to the presence of these autophagic structures and residual bodies associated with senescence.

7.3.2 Measuring the synthesis of subunit *c* in cultured kidney cells

The presence of structures that were interpreted to resemble lipopigment bodies in some affected cells suggested the possibility of being able to investigate the metabolism of subunit *c* from affected cultured cells. Synthesis of subunit *c* in affected and control cells was investigated by exposing confluent cell monolayers to a ^{3}H amino acid mixture. Added label was incorporated into TCA precipitable cellular protein to a maximal value of 30% after a 2 h incubation. Rates of incorporation were similar in both affected and control monolayer cells (Fig. 7.5).

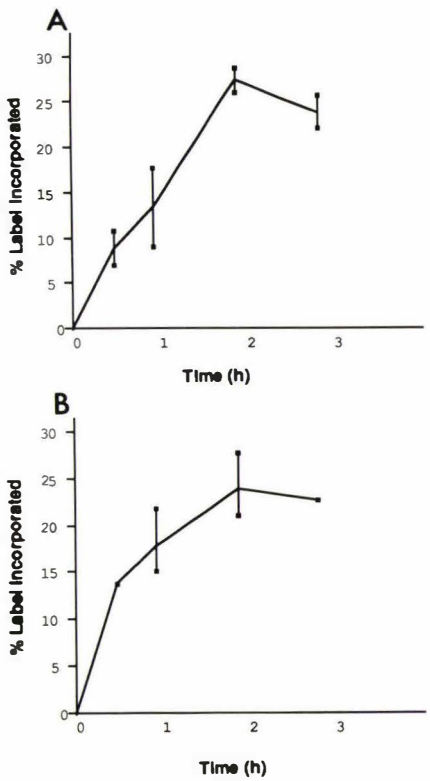

Fig.7.5 % of ^{3}H incorporated into TCA precipitable protein from labelled affected (A) and control (B) primary kidney cell cultures. Each point on the graphs represents determinations from monolayer cells harvested from 25 cm^2 tissue culture flasks. The radiolabelling procedure and detection of added label is described in section 7.2.5.

Incorporation of ^{3}H into proteolipids of affected and control cells labelled for 3 h and "cold chased" in maintenance media for 24 h prior to harvesting was determined next. There was no significant differences in the specific activities (dpm / mg of protein) of proteolipids extracted from affected and control cells (Table 7.1).

Table 7.1 The specific activities (dpm/mg of protein) of the proteolipid fractions extracted from five affected and five control cell cultures. Protein recovered by chloroform/methanol/ammonium acetate extraction was of the order of 2.5 μg/mg of cell protein harvested from each 25 cm^2 culture flask.

	Affected cell culture proteolipid fractions	Control cell culture proteolipid fractions
1.	$2.0x10^5$	$2.4x10^5$
2.	$1.8x10^5$	$2.0x10^5$
3.	$2.2x10^5$	$1.3x10^5$
4.	$2.5x10^5$	$2.4x10^5$
5.	$2.1x10^5$	$1.7x10^5$

Separation of the proteolipid fractions by LDS-PAGE showed that the radioactivity was distributed amongst the higher molecular weight bands with no activity in the region that subunit *c* is known to migrate, in both affected and control preparations (Fig. 7.6 A & C). The addition of 0.2 vol of lipopigment in chloroform/methanol/ammonium acetate prior to ether precipitation of the proteolipid fraction made no difference to the ^{3}H distribution (Fig. 7.6 B & D).

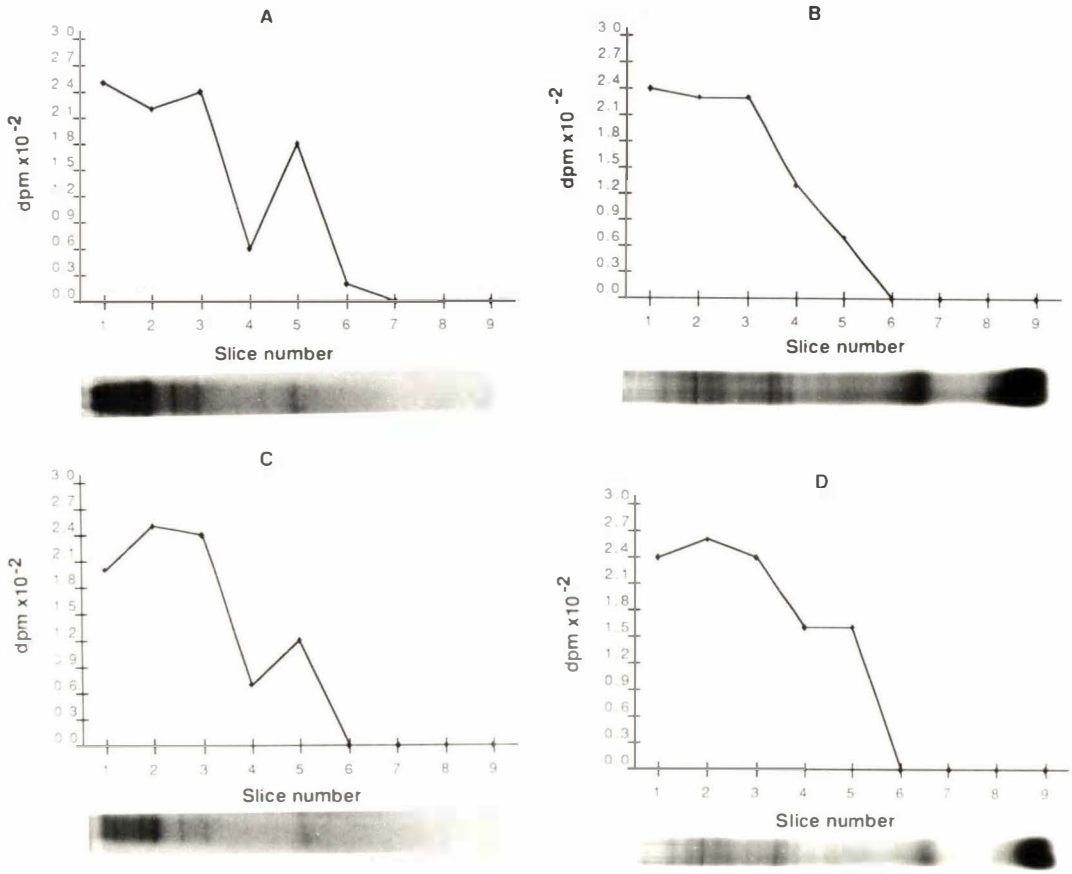

Fig.7.6 ^{3}H distribution amongst the proteolipid fractions separated by LDS-15% PAGE isolated from affected and control primary cell cultures (A and C, respectively). The distribution of label after the addition of a carrier prior to proteolipid extractions from affected and control cells (B and D, respectively). The radiolabelling procedure used is described in section 7.2.5.

7.4 DISCUSSION

Ultrastructural features that were interpreted to resemble lipopigment bodies in tissue sections were seen in some affected primary cultured cells and early passaged cells at confluence. They were not seen in control cell cultures. The presence of these structures in the presence or absence of antibiotics in the media indicated that they were unlikely to be lamellar bodies that can sometimes be induced in cultured cells by amphiphilic drugs (Joshi, *et al.*, 1989). The small amounts present however, indicated that this lesion was minimally expressed in tissue cultures. Rapid cell proliferation over comparatively short time periods would also have militated against levels of storage that would normally have occurred over a longer time period *in vivo*, (i.e. rapid cell proliferation would result in division of the stored material quicker than its storage). The small numbers of lipopigment structures may also explain the failure of the luxol fast blue to detect these in affected cultured cells.

Primary and passaged cells reaching confluence were chosen for the labelling experiment as they were considered to most closely resemble cells under steady state conditions. Variations in cell numbers from each flask of monolayer cells could also be minimized by the use of cells reaching confluence. Total cellular proteolipid fractions were extracted on the assumption that if there was abnormal synthesis of the proteolipid subunit *c* in affected cells, then it should have been reflected by an increase in incorporation of the added label into this fraction compared to that in control fractions. Although no differences were detected under the conditions of the experiment, no conclusions can be drawn from this result as subsequent separation of the extracted proteolipid fractions by LDS-PAGE failed to show the presence of labelled subunit *c*, (i.e there was no evidence that labelled subunit *c* was synthesized during the labelling period). The small amounts of protein harvested from each flask of confluent monolayer cells suggested the possibility that failure to detect subunit *c* may have been due to incomplete ether precipitation of the proteolipid. However, this possibility was eliminated as addition of sufficient carrier in the form of lipopigment dissolved in chloroform/methanol/ammonium acetate to ensure ether precipitation of proteolipids, made no difference to the radioactive distribution of proteolipid proteins separated by LDS-PAGE (Fig. 7.6).

The presence of cytoplasmic structures that were interpreted to resemble lipopigment bodies seen in tissue sections in cultured epithelial cells, indicated that the kidney tissue culture system has potential to be developed further as an *in vitro* model of ovine ceroid-lipofuscinosis.

Longer exposure of confluent cell monolayers to the amino acid deficient medium and longer incubation with the added label in the presence of agents such as insulin and epidermal growth factor that selectively stimulate protein synthesis (Ballard, 1982) might improve the levels of label incorporated into cellular proteins (Fig. 7.5). Subcellular fractionation to isolate subunit *c* in its normal cellular location (inner mitochondrial membrane) could also be investigated to study the turnover rates of this protein. The use of a label with higher specific activity such as ^{35}S methionine may also help with better detection.

Extraction of subunit *c* from mitochondrial fractions isolated from kidney cells cultured in the presence of radiolabelled amino acids could provide a means of obtaining a potential labelled substrate for further experimentation. A major problem encountered during this study was the relatively small number of kidney epithelial cells that were initially able to be cultured. Although successive passaging provided a way to overcome this, cells passaged a number of times will ultimately result in the proliferation of fibroblasts cells in which disease specific lesions are not known to occur (Graydon and Jolly, pers. comm.). The development of a stable defined cell line that shows disease specific lesions is highly desirable. A possibility that could be considered would be to immortalize the primary culture by nonviral or viral mediated transformation similar to that achived with human epithelial cells (Stampfer and Bartley, 1985; Chang, 1986).

CHAPTER 8

ISOLATION AND CHARACTERISATION OF LIPOPIGMENT FROM A CASE OF BOVINE CEROID-LIPOFUSCINOSIS.

8.1 INTRODUCTION

Ceroid-lipofuscinoses, analogous to that described in South Hampshire sheep and humans, have been described in a number of animal species (Chapter 1). This chapter describes the isolation and protein analysis of lipopigment from frozen tissue of an affected 18 month old heifer from a Devon herd in New South Wales, Australia in which ten other cases of the disease had occured over a 10 year period. The disease was characterised by severe retinal degeneration and the intracellular accumulation of lipopigment characteristic of the ceroid-lipofuscinoses in a wide variety of cell types throughout the body (Harper, *et al.*, 1988).

8.2 SPECIAL MATERIALS AND METHODS

8.2.1 BOVINE TISSUE

Frozen brain, pancreas, liver and kidneys were obtained from an 18 month old Devon heifer euthanased in the terminal stages of ceroid-lipofuscinosis. They were stored at -20°C until used. Diagnosis was confirmed by histopathological examination of tissues (P.A.W. Harper, pers. comm.).

8.2.2 ISOLATION OF BOVINE LIPOPIGMENT BODIES

Lipopigment enriched pellets were obtained from frozen Devon pancreas, liver, brain and kidney tissues by the same combination of homogenisation, osmotic shock, sonication, and differential centrifugation steps used for the ovine preparations (section 2.2).

Lipopigments suspended in deionized water were purified further by isopycnic centrifugation on 15+15 ml linear 0.64-3.38 M caesium chloride (CsCl) density gradients at 141,000 g_{max}, 24 h at 4°C in a Beckman SW 28 swing out rotor on a Beckman LB-70 Ultracentrifuge. The lipopigment bands were identified by their fluorescence when irradiated with 366 nm ultraviolet light. The fluorescence was photographed through a yellow filter on Ektachrome 64 ASA film. The lipopigment bands were removed from the gradients and their densities determined by measuring the refractive index of the CsCl solutions (Weast, *et al.*, 1988). They were then washed twice with deionized water by resuspension and centrifugation at 48,000 g_{max} for 10 min. An aliquot was taken for thin section electron microscopy as described in section 2.3. The remaining pellet was resuspended in deionized water and stored at -20 °C until required.

8.2.3 CYANOGEN BROMIDE DIGESTION OF ISOLATED LIPOPIGMENT

Total pancreatic lipopigment dissolved in distilled 98% formic acid was treated with cyanogen bromide as described in section 5.2.2.

8.3 RESULTS

8.3.1 Characteristics of bovine lipopigments

In contrast to the isolation of lipopigment from fresh ovine tissues, a CsCl isopycnic centrifugation step was required to achieve comparable purification from the frozen bovine tissues. Thin section electron microscopy of isolated lipopigments showed that they were relatively free of other subcellular organelles or debris, and similar in structure to those seen *in situ* (Fig. 8.1).

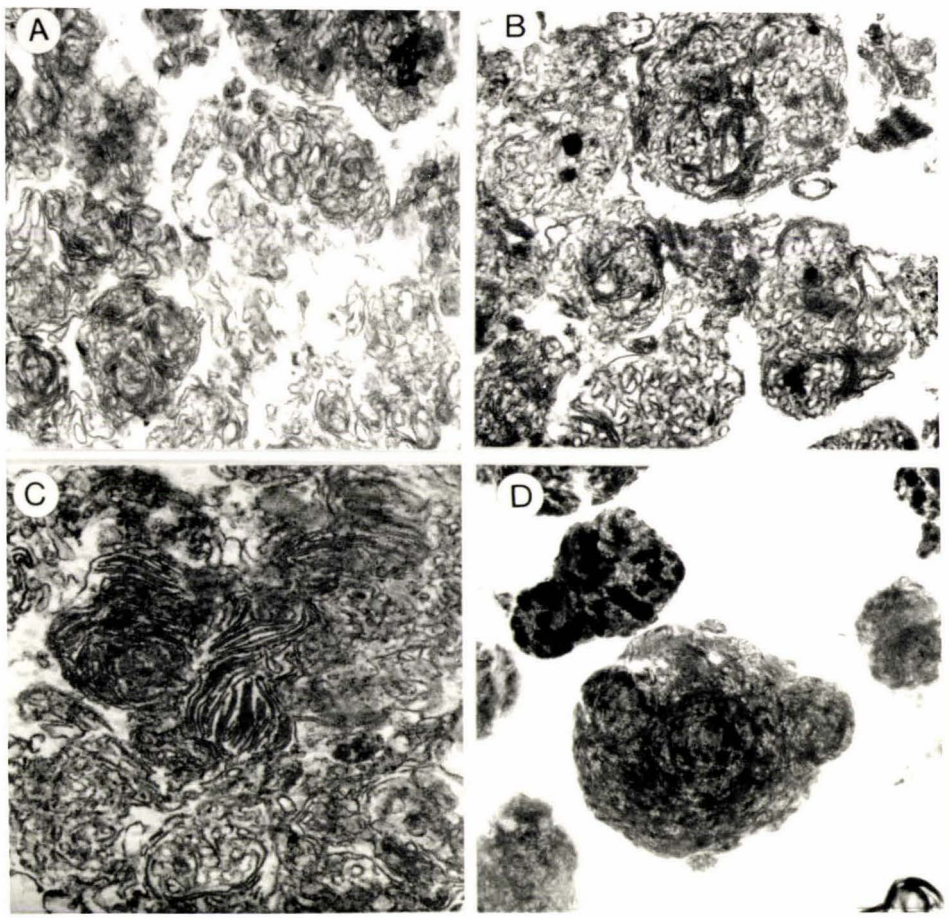

Fig. 8.1 Thin section electron micrographs of lipopigment isolated from bovine pancreas (A), liver (B), kidney (C) and brain (D). Magnification x 21,000.

Lipopigment bodies were isolated from frozen pancreas, liver, kidney and brain tissues in yields of 0.6-1.8 mg of dry weight /g wet weight of tissue. Their protein contents, determined by quantitative amino acid anaylsis of hydrolysed lipopigment, accounted for 55-62% of the total lipopigment dry masses (Table 8.1).

Table 8.1 Characteristics of lipopigment isolated from bovine tissues.

Tissue	Yield[a]	Density[b]	Protein[c] (% of the dry mass of total lipopigment bodies)
Pancreas	1.8	1.17-1.19	59
Liver	1.7	1.2-1.24	60
Kidney	0.8	1.2-1.24	62
Brain	0.6	1.15	55

a. mg dry weight of isolated lipopigment/g wet weight of tissue.

b. determined by isopycnic centrifugation on CsCl gradients (0.64-3.38M).

c. determined from the amino acid content after hydrolysis. Mean of three estimations.

The isolated lipopigment exhibited a yellow fluorescence when irradiated with 366 nm ultraviolet light (Fig. 8.2). Isopycnic densities of isolated lipopigment bodies ranged from 1.15-1.24, depending on their tissue of origin (Table 8.1).

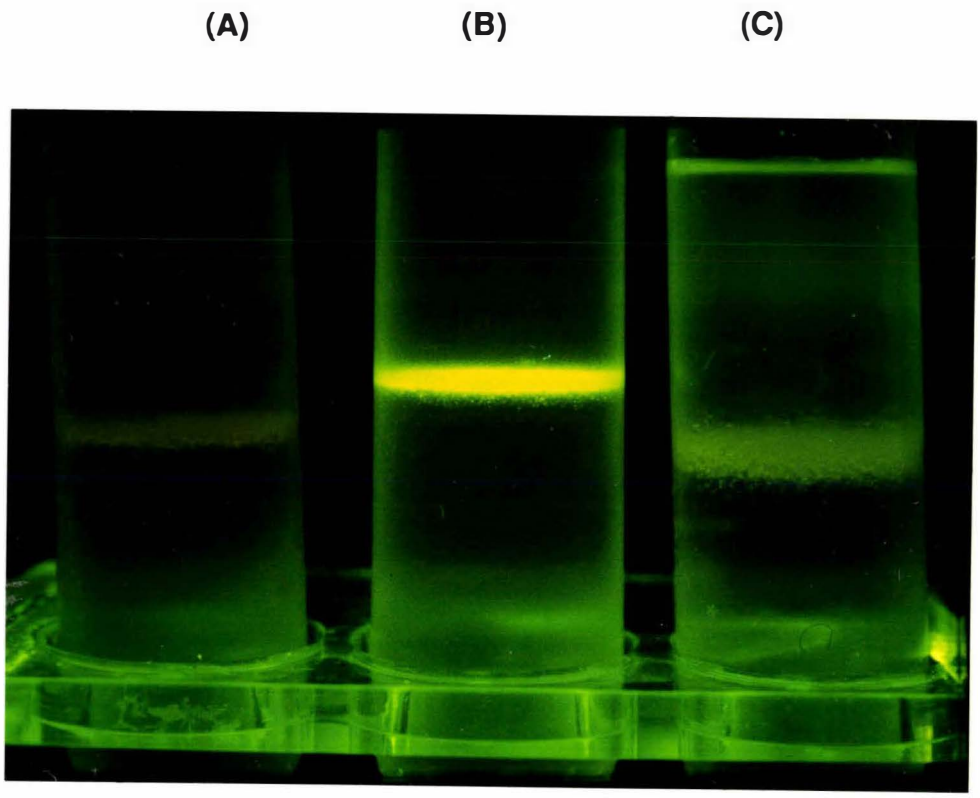

Fig. 8.2 Isolated bovine lipopigments on CsCl isopycnic gradients when irradiated with 366 nm ultraviolet light from; kidney (A), pancreas (B) and liver (C).

8.3.2 Amino acid composition and LDS-PAGE of bovine lipopigment proteins

The amino acid compositions of lipopigments from brain, pancreas, kidney and liver showed a high content of alanine, glycine, and leucine residues (Table 8.2).

Table 8.2 The amino acid composition, in moles % of bovine lipopigments compared with the full subunit *c* of mitochondrial ATP synthase.

	Total lipopigment				Extracted proteolipid	subunit c[a]
Amino acid	Brain	Pancreas	Kidney	Liver[b]	Pancreas[c]	
Asp	5.3	5.0	8.5	5.2	4.3	4.0
Thr	5.6	6.4	5.4	5.0	4.6	4.0
Ser	6.1	5.6	6.0	6.9	6.4	6.7
Glu	5.2	3.2	8.0	5.0	5.2	4.0
Pro	2.3	2.4	5.0	2.9	3.8	1.3
Cys	ND[d]	ND	ND	ND	ND	1.3
Gly	12.2	10.9	9.1	11.6	12.3	14.7
Ala	12.5	13.3	8.7	11.7	15.0	17.3
Val	8.2	8.9	8.2	9.3	6.2	5.3
Met	3.3	2.6	2.0	3.0	2.8	4.0
Ile	8.8	9.4	6.4	8.0	8.2	9.3
Leu	12.3	11.4	11.4	11.0	11.8	12.0
Tyr	3.1	4.8	3.1	2.9	3.5	2.7
Phe	7.4	7.3	6.0	7.8	8.5	9.3
His	2.0	1.7	2.4	1.5	0.9	0
Lys	3.4	3.7	5.3	3.6	3.0	2.7
Arg	2.3	3.4	4.5	4.6	3.5	1.3

a. Values calculated from the cDNA sequence of the full bovine protein (Gay and Walker 1985).

b. Mean of three estimations.

c. 66% of total lipopigment protein extracted as proteolipids, mean of two estimations.

d. Not determined.

LDS-PAGE of the lipopigment protein showed the presence of a major band, that comigrated with the 3.5 kDa band in ovine pancreatic lipopigment, and another band that comigrated with the 14.8 kDa ovine lipopigment component (Fig. 8.3).

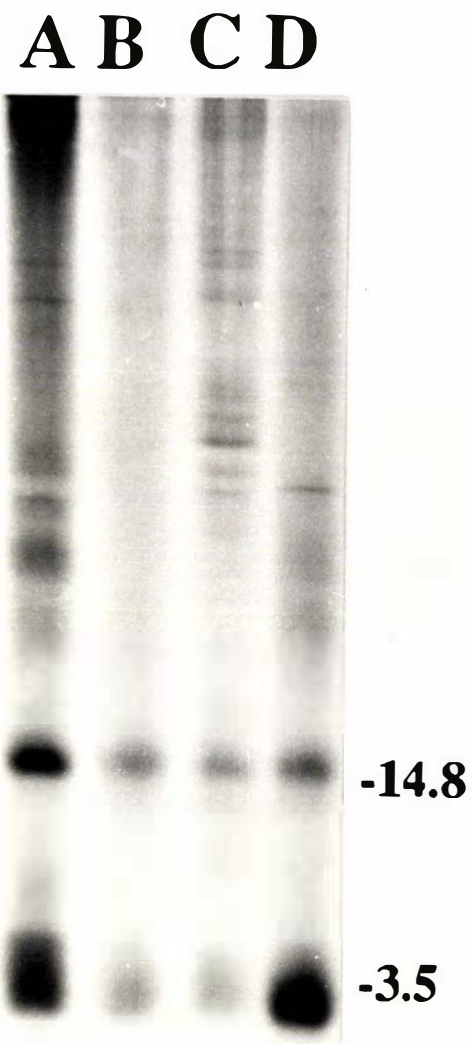

Fig.8.3 Silver stained LDS-15% PAGE of lipopigment isolated from ovine pancreas (A) and bovine liver, kidney and pancreas (B, C and D, respectively), (15 µg/lane). The numbers on the right hand side indicate apparent molecular weights calculated from the migration of molecular weight markers.

8.3.3 Amino acid sequencing of bovine lipopigment

Protein sequencing was carried out as described in section 4.2.2, except that total lipopigment rather than lipopigment protein was loaded onto the sequencer. A dominant sequence was obtained from pancreatic lipopigment, which was determined to 25 residues with a repetitive yield of 90% (Fig. 8.4). This sequence was identical with the first 25 amino-terminal residues of subunit *c* of mitochondrial ATP synthase. The same amino-terminal sequence was obtained from both liver and brain derived lipopigment and confirmed to 15 and 20 residues respectively.

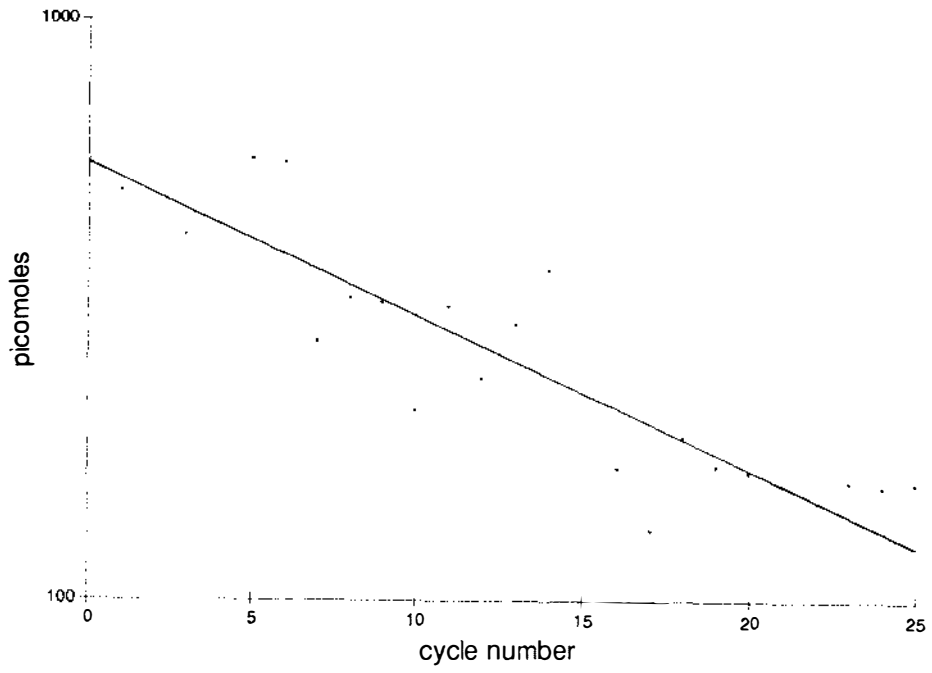

Fig.8.4 The PTH amino acid yields of the first 25 residues of subunit *c* obtained when bovine pancreatic lipopigment containing 61.4×10^{-9} moles of hydrolysable amino acids were sequenced. The molar quantity of the peptide that coupled, 0.56×10^{-9} moles, was read from the intercept of the repetitive yield slope with the y-axis. The estimate of the contribution of subunit *c* to the total lipopigment protein was made by multiplying the initial yield 0.56×10^{-9} by 75 (the length of the stored protein)= 41.7×10^{-9} divided by the total protein loaded.

i.e $$\frac{41.7 \times 10^{-9}}{61.4 \times 10^{-9}} \times 100 = 68\%$$

8.3.4 Mass spectral analysis of the CNBr digest of bovine lipopigment

When the products of cyanogen bromide digestion of total pancreatic lipopigment were analysed by liquid secondary ion mass spectrometry, a major peak with a mass of 994.5984 and a peak at 976.5573 Da were observed. These masses were consistent with those expected for the homoserine and homoserine lactone forms of the carboxyl-terminal fragment of subunit *c* arising from cleavage at Met^{66} (calculated as 994.5977 and 976.5871 Da respectively) (Fig. 8.5). This result indicated that the full subunit *c* was likely to be present in the isolated bovine lipopigment.

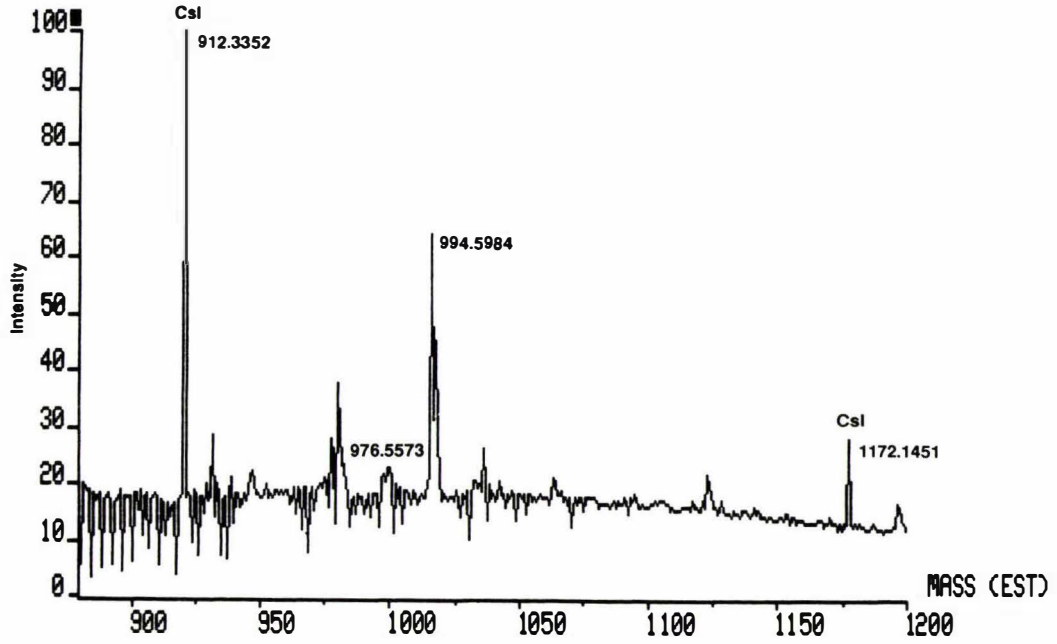

Fig.8.5 Linear mass spectral scan over the molecular ion region of the carboxyl-terminal CNBr cleavage fragment of subunit *c* isolated from bovine pancreatic lipopigment. CsI calibrant signals were used for the mass measurement of the peptide fragment.

8.4 DISCUSSION

Bovine lipopigment exhibited the same properties of resistance to osmotic shock and sonication as the lipopigment isolated from sheep affected with ceroid-lipofuscinosis. Isolated lipopigments from various tissues had densities similar to those determined for ovine lipopigments (Palmer, *et al.*, 1986b). They had high protein contents (55-62%) and contained the same low molecular weight protein bands with apparent molecular weights of 3.5 and 14.8 kDa found in ovine lipopigment.

The sequencing approach described in Chapter 4 was used, except that the size exclusion HPLC step was omitted and total lipopigment rather than lipopigment protein was loaded onto the sequencer. The presence of the first 25 amino-terminal residues and the expected carboxyl-terminal cyanogen bromide digest fragment of the full subunit *c* indicated that the full protein was likely to be present in bovine lipopigment. Although residues 26-66 were not confirmed by direct sequencing, the amino acid compositions of the extracted proteolipid (Table 8.2) are consistent with the complete molecule being stored in lipopigment. Additional evidence that the complete molecule is stored was provided by the LDS-PAGE comigration and similar staining properties to subunit *c* stored in the ovine disease (Fig. 8.3). Quantitation of the sequence data showed that the full subunit *c* of mitochondrial ATP synthase accounted for at least 68% of the lipopigment protein (Fig. 8.4). This agrees with the 66% of the lipopigment that was shown to extract as proteolipid. Both these are minimum estimates as the initial reactions in the sequencer are not quantitative nor were the proteolipid extractions exhaustive. Similar calculations showed that subunit *c* accounted for 38% of the brain and 22% of liver lipopigment protein. As proteins account for 59% of total pancreatic lipopigment, subunit *c* alone accounts therefore, for at least 40% of the total lipopigment mass. This is similar to that found in the ovine disease, where subunit *c* was shown to account for at least 50% of the total lipopigment mass (Chapter 5). These results indicate that both the ovine and bovine ceroid-lipofuscinoses lead to the accumulation of subunit *c* in lysosome derived lipopigment bodies.

CHAPTER 9

CANINE CEROID-LIPOFUSCINOSIS

9.1 INTRODUCTION

Ceroid-lipofuscinosis is described in a number of canine breeds (Chapter 1). Many such descriptions are however, case reports and require further study to define them more precisely. The English Setter has been extensively studied, and with the exception of retinal degeneration, has been shown to resemble the juvenile human disease (Koppang, 1973/74; 1988). Lipopigment bodies isolated from the English Setter have been shown to contain subunit *c* (D.N. Palmer, unpublished).

In this chapter lipopigment bodies isolated from frozen brain from a 2 year old Border Collie and a 9 year old Tibetan Terrier, two breeds having distinct forms of the disease, were analyzed for the storage of subunit *c*. In the former breed, clinical signs of neurological disease developed before 2 years of age and progressed rapidly with the storage of lipopigment bodies and brain atrophy similar to that described in the English Setters and other canine breeds (Taylor and Farrow, 1988). The disease in the Tibetan Terrier, however, was distinctive in that affected animals showed signs of night blindness and only slight neurologic deterioration after a number of years. It is thought to represent an adult form of ceroid-lipofuscinosis somewhat similar to Kufs disease of humans (Riis, *et al.*, 1990).

9.2 SPECIAL MATERIALS AND METHODS

9.2.1 CANINE TISSUES

Frozen brains from a 2 year old Border Collie and a 9 year old Tibetan Terrier euthanased in the terminal stages of ceroid-lipofuscinosis were obtained from V. Studdert (University of Melbourne, Australia) and R. Riis (Cornell University, U.S.A.) respectively. They were transported in liquid nitrogen and stored at -20°C until used.

9.2.2 ISOLATION OF CANINE LIPOPIGMENT BODIES

Lipopigment was isolated from the frozen brain tissues and investigated by the methods described in section 8.2.2.

9.3 RESULTS

9.3.1 Characteristics of canine lipopigment

Isolated lipopigment bodies from the Border Collie frozen brain tissue showed the presence of membraneous electron dense granular structures similar to those observed from isolated ovine and bovine lipopigment bodies (Fig. 9.1 A). The Tibetan Terrier lipopigment was more heterogeneous in appearance. Electron dense granular structures and characteristic membraneous whorls similar to those found in sections of neurones and macrophages of affected brain (R.D. Jolly, pers. comm.) were found (Fig. 9.1 B & C).

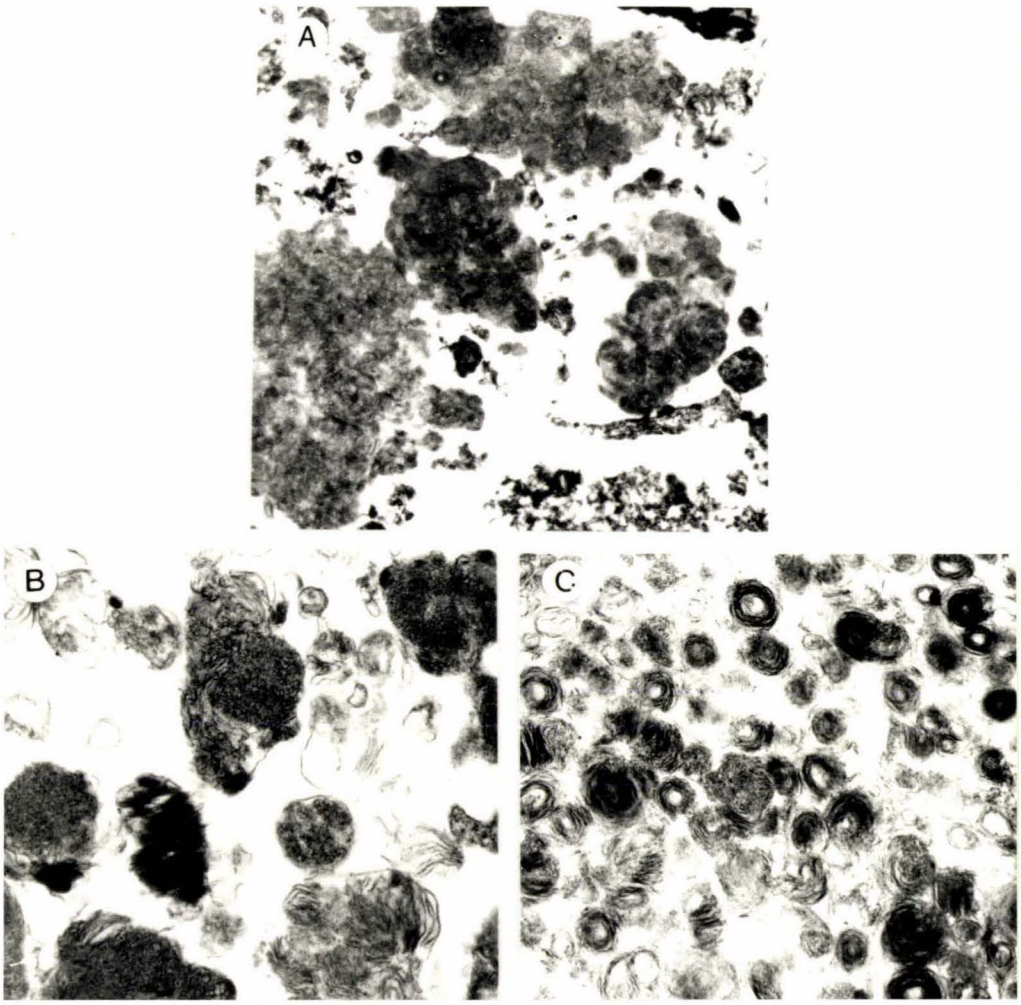

Fig.9.1 Thin section electron micrographs of lipopigment isolated from frozen Border Collie brain (A) and Tibetan Terrier brain (B and C). Magnification x 21,000.

These structures were observed amongst subcellular debris which indicated that the isolated preparations were not as homogeneous as those from ovine and bovine tissues described in previous chapters. Insufficient material precluded further purification by a second CsCl isopycnic density centrifugation step. However, sufficient lipopigment bodies were present to allow further analysis.

9.3.2 Amino acid sequencing of canine lipopigment

When aliquots of the brain lipopigment dissolved in formic acid were loaded onto the protein sequencer as described in section 4.2.2, a sequence identical to that of the first 16 amino-terminal residues of subunit *c* were detected in preparations from both breeds. A high background precluded further sequencing beyond 16 cycles (Fig. 9.2). The sequence, **Gly-Pro-Glu-Tyr**, detected previously in ovine pancreatic lipopigment, was also able to be assigned in the first few cycles from the Tibetan Terrier lipopigment preparation.

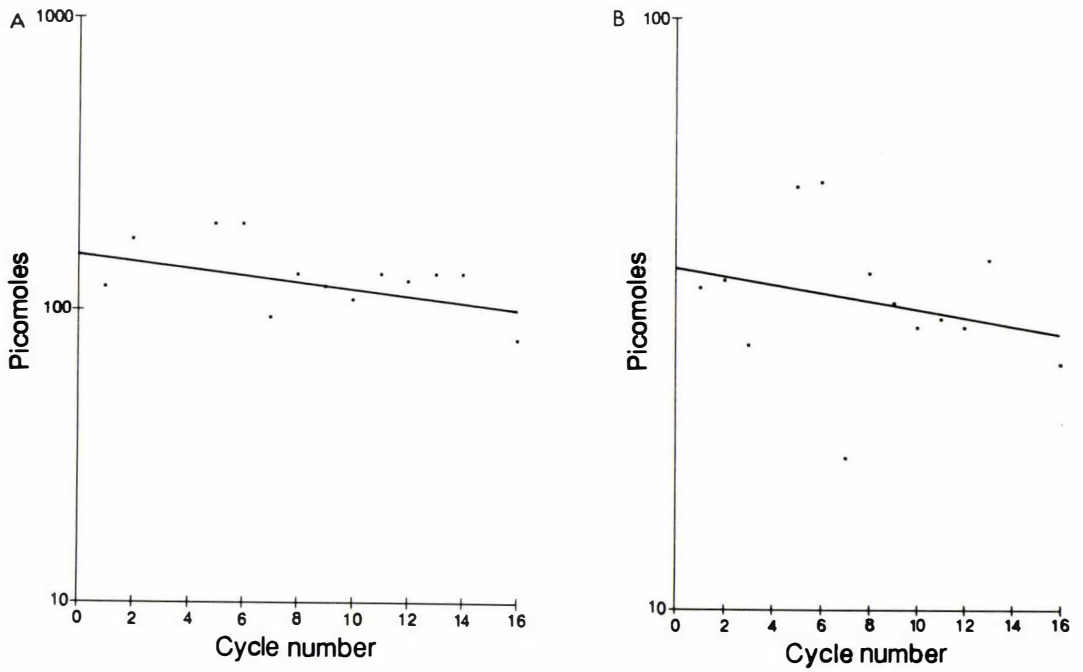

Fig.9.2 PTH amino acid yields of the first 16 amino-terminal residues of subunit *c* obtained from the Border Collie (A) and Tibetan Terrier (B) lipopigment. Initial yields were read from the intercept of the repetitive yield slope with the y-axis.

9.4 DISCUSSION

The presence of the 16 amino-terminal residues of subunit *c* in lipopigment isolated from the two canine breeds suggested that they also belonged to the forms of ceroid-lipofuscinoses that specifically store subunit *c*. The molar quantity of subunit *c* that coupled was determined as before, from the repetitive yield graphs of the PTH amino acids obtained at each cycle (Fig. 9.2). If the full 75 residue protein was stored, estimates of 55% and 33% were calculated for the contribution of subunit *c* to the total lipopigment masses from the Border Collie and Tibetan Terrier, respectively. These estimations are minimum values because of relative impurity of preparations and the fact that the initial reactions in the sequencer are not quantitative. They are also based on the assumption that the full protein is stored as in the ovine and bovine forms of the disease (Chapters 5 & 8). Further characterisation of the stored protein was not possible due to the limited amount of material available.

As the development of modern breeds within a species is relatively recent it is likely that some forms of the disease within the same species may reflect the same mutation. In this regard, the diseases in the Border Collie (Taylor and Farrow, 1988) and Blue Heeler dog (Cho, *et al.*, 1986; Wood, *et al.*, 1987) have been shown to be clinically and pathologically similar to the English Setter disease (Koppang, 1973/74; 1988), and may reflect the same mutation. The disease in Tibetan Terrier is however, distinctively different (Riis, *et al.*, 1990).

The finding of two distinct diseases in dogs which both store at least an amino-terminal fragment of subunit *c* in lipopigment, indicates two distinct mutations. Whether these affect the same gene or different genes may be determined by cross breeding experiments. If the same gene is mutated then affected offspring should have a disease with characteristics between the parent types. If no disease results from the cross breeding then involvement of a second mutant gene is implied. The different human forms of the disease could reflect similar mutations.

CHAPTER 10

GENERAL DISCUSSION

Inborn errors of metabolism are reflected by elevation of the deficient enzyme's substrate or derivatives of it, a principle first enunciated by Garrod in 1908. In the case of storage diseases, characterisation of the dominantly stored species has led in many instances to elucidation of the underlying biochemical defect. The same principle should therefore be expected to hold for the ceroid-lipofuscinoses. Palmer, *et al.*, (1986a,b) showed that in the ovine disease the major stored components were low molecular weight proteins in lysosome derived lipopigment bodies. Characterisation of these proteins by a combination of LDS-PAGE, quantitative amino acid sequencing and mass spectroscopy studies has led to the major stored component being identified as the complete dicyclohexylcarbodiimide (DCCD) reactive proteolipid (subunit *c* of the mitochondrial ATP synthase). This is a highly hydrophobic protein normally found in the inner mitochondrial membrane as part of the ATP synthase oligomeric protein complex. The ATP synthases (also known as proton translocating ATPase, H^+-ATPase, or F_1F_0-ATPase) are found in the cytoplasmic membranes of eubacteria, the thylakoid membranes of chloroplasts and in the inner membranes of mitochondria. Their common function is to catalyze the synthesis of ATP from ADP and phosphate by employing an electrochemical potential gradient across the membrane. The mitochondrial ATP synthase is a complex of 14 different polypeptides; six of them (α, β, g, σ, ε, OSCP and I) constitute F_1, the extrinsic membrane domain of the complex. The remainder (ATPase-6, subunit *c*, subunit *b*, subunit *d*, A6L) make up the membrane sector F_0. Two additional components, subunit *e* and F6 are also found (Walker, *et al.*, 1990). Subunit *c*, is an essential element involved in the H^+ translocating properties of F_0 and accounts for 2-4% of the inner mitochondrial membrane protein (Sebald and Hoppe, 1981).

Solubility of the lipopigment proteins in LDS, in the presence or absence of 2-mercaptoethanol was important as it showed that the aggregation

behaviour of these proteins in dodecyl sulphate was not due to the presence of covalent disulphide bonding between them. The difficulty of separating the major 3.5 kDa lipopigment protein from the other lipopigment proteins led to the non-traditional approach of loading an apparent mixture of proteins onto the amino acid sequencer (Chapter 4). It was reasoned that should a sequence emerge, then it would be of the low molecular weight component shown in Chapter 3 to be present in total lipopigment protein as the dominant molar component. The approach led to the detection and quantitation of subunit *c* in lipopigment. Identification of the 14.8 and 24 kDa lipopigment proteins as being oligomers of the major stored protein (subunit *c*) strengthened the rationale used in the earlier sequencing studies on total lipopigment proteins. This result explained the difficulties encountered in separating the 3.5, 14.8 and 24 kDa proteins by conventional techniques. Subunit *c* was therefore the major protein present in lipopigment bodies. No other mitochondrial protein was detected during this study.

The aggregation behaviour of the proteolipid subunit *c*, particularly in dodecyl sulphate, is well documented (Sebald and Hoppe, 1981). As noted earlier the aggregation behaviour of subunit *c* stored in lipopigment was not due to the presence of intermolecular disulphide bonding. The unusual stability of the oligomers of subunit *c* in lipopigment was shown by the 14.8 kDa protein that diffusion eluted from a polyacrylamide gel behaving as a component distinct from the 3.5 kDa protein (Chapter 5). The physico-chemical basis for this oligomeric stability remains unknown. The electrophoretic variability of the 14.8 and 24 kDa bands observed between different isolates and between different gels of the same isolate could now be rationalized on the basis of variable aggregation of subunit *c* present. The behaviour of the ^{125}Iodinated lipopigment proteins on storage described in Chapter 3 was also probably due to this aggregation property of subunit *c*. The radioiodination of lipopigment proteins as a means of obtaining a labelled substrate therefore warrants further investigation.

The stored protein was shown to account for at least 50% of the isolated ovine lipopigment mass, and as such was considered specific to the

disease. Its relation to the other components is shown in Table 10.1, which is derived from Palmer,*et al.*, 1986b; 1988; Hall,*et al.*, 1989, and the present study. As noted in Chapter 1, the lipid and metal contents of lipopigments were considered to be consistent with their lysosomal origin.

Table 10.1 Ovine pancreatic lipopigment composition

Components	(%)
Subunit *c*	50
Other proteins	20
Phospholipids	10.5
Neutral lipids	10.5
Metals	1.3
Dolichol-P-P-Oligosaccharides	2
Other	5.7

The sequencing approach used in this study has proved to be extremely robust. The amino-terminal sequence of subunit *c* can be determined even when total lipopigment bodies dissolved in formic acid are loaded onto the amino acid sequencer. There is thus available a relatively simple method for determining whether a disease, diagnosed on morphological grounds as ceroid-lipofuscinosis, is associated with storage of subunit *c*. Application of this methodology has shown two distinctly different canine forms and a bovine form of the disease to store at least an amino-terminal fragment and the complete subunit *c*, respectively (chapter 8 and 9).

In parallel studies Palmer, *et al.*, (1990) showed that the same protein is also stored in the late infantile and juvenile human diseases but not in the infantile disease. This latter result demonstrated that not all diseases falling within the general classification of ceroid-lipofuscinosis are closely related.

The described methodology could be used to determine the dominantly stored components from the other human variants and animal forms of the disease. Once lipopigment is isolated from subcellular organelles and debris, only four aliquots of isolated lipopigment are required; one for electron microscopy to determine purity of preparation, two for quantitative amino acid analysis and amino-terminal sequencing and one aliquot for cyanogen bromide cleavage to determine the carboxyl-terminus of subunit *c*. It is likely that a classification of subunit *c* storage disease could be made from the mere demonstration of the amino-terminus of subunit *c* by direct sequencing studies, when there is insufficient material for the other analyses. Such was demonstrated in the the two canine diseases (Chapter 9).

The storage of subunit *c* rather than peroxidised lipid/protein polymers as previously postulated, allows a major conceptual change in the approach to ceroid-lipofuscinosis research. Histochemical and fluorescent properties of the stored lipopigment which were interpreted to be due to their lipid nature, led to a number of theories on pathogenesis, most based on lipid peroxidation (Chapter 1). None of these have stood the test of time or led to the elucidation of the underlying biochemical defect in the ceroid-lipofuscinoses. An "acidic lipid polymer" extracted from lipopigments by lipid solvents and named "ceroid" was proposed as the "stored substance" in the neuronal ceroid-lipofuscinoses (Siakotos, *et al.*, 1972). In retrospect, it seems likely that the designated " acidic lipid polymer" was the proteolipid, subunit *c*.

The extreme hydrophobic nature of subunit *c* results in it having unusual physical properties, including the lipid like solubility in chloroform/methanol mixtures. It is this property which results in it being referred to as a proteolipid. The definition is an operational one and does not imply the presence of covalently bound lipids. Subunit *c* stains poorly with Coomassie blue and gives inaccurate protein estimations by the Lowry method (Sebald and Hoppe, 1981). These characteristics may help explain why this type of protein was not recognized as being associated with the ceroid-lipofuscinoses in the past.

Many characteristics of the stored lipopigment bodies and other enigmatic observations can now be explained by the knowledge that it is subunit *c* that is stored. These include the characteristic histochemical feature of staining with certain lipid stains. Lipid stains can be expected to react with the neutral lipids and phospholipids present in lipopigment bodies but may also react with the hydrophobic proteolipid subunit *c*. Luxol fast blue, considered a stain for phospholipids and phospholipid/protein complexes in fixed tissues (Pearse, 1985), is also used in neuropathology as a stain for myelin. As myelin contains the prototype proteolipid protein (Folch and Lees, 1951) it is likely that it is this type of molecule that stains both myelin and lipopigment bodies in paraffin block sections (Jolly, *et al.*, 1989). This is supported by the fact that luxol fast blue staining is a feature of all but the infantile human form of ceroid-lipofuscinosis (Lake, 1984) in which subunit *c* is not stored (Palmer, *et al.*, 1990). The luxol fast blue staining may therefore have some diagnostic importance.

Autofluorescence in the yellow-orange region (520-540 nm) is another characteristic feature of stored lipopigment. To date neither the nature of the flourophor nor its significance, if any, has been determined. A number of mechanisms whereby proteins can be modified to form fluorophores have been proposed. These include peroxidation reactions leading to the formation of malonaldehyde, which can react with protein to form fluorescent schiff base products (Chio and Tappel, 1969a) and nonenzymic glycation (glycosylation) of lysine residues producing heterocyclic fluorescent compounds (Pongor, *et al.*, 1984). There has however, been no evidence for the presence of such compounds in lipopigment bodies isolated from the ceroid-lipofuscinoses. Palmer, *et al.*, (1986a) suggested that fluorescence may be due to a property of the interaction of the stored protein and its peculiar lipid environment. This idea is supported by reconstitution of fluorescent bodies from nonfluorescent purified subunit *c* and phospholipids (D.N. Palmer, pers. comm.).

Detection of S-methylated methionine in "storage protein" isolated from the late infantile form of the human disease has recently been reported

(Katz and Gerhardt, 1990). It was suggested that such modified amino acids might be responsible for the autofluorescence of storage bodies under uv illumination. However, the amino acid compositions of the protein isolated from lipopigment in the study differs substantially from that of subunit *c*, identified as the major stored species in the late infantile disease (Palmer, *et al.*, 1990). The significance of this observation in terms of a possible post-translational modification to subunit *c* stored in the ceroid-lipofuscinoses remains to be clarified. There was however, no evidence in the present studies to suggest that the stored protein in the ovine, bovine and canine forms of ceroid-lipofuscinosis was post-translationally methylated.

The presence of large amounts of the complete and apparently unmodified subunit *c* in the ceroid-lipofuscinoses indicates that the biochemical basis of the lesion in these diseases is likely to involve the metabolism of subunit *c* .

In humans and cattle, subunit *c* has two expressed nuclear genes, P1 and P2. These encode precursor proteins containing the same mature protein, but with different positively charged amino-terminal extensions (presequences) of 61 and 68 amino acids respectively (Gay and Walker, 1985; Dyer, *et al.*, 1989). In addition to the P1 and P2 genes, numerous spliced and partly spliced pseudogenes related to P1 or P2 have also been discovered in human, bovine and ovine tissues (Dyer and Walker, 1990; Medd, *et al.*,unpublished).

The presequences contain the necessary information to target the protein to mitochondria (van Loon, *et al.*, 1987; van Loon and Schatz, 1987; Hartl, *et al.*, 1986; 1987). Import receptors on the outer surface of the outer mitochondrial membrane are involved in the initial recognition process of these precursors (Zwizinski, *et al.*, 1984; Pfaller, *et al.*, 1988; Pfaller and Neupert, 1987). It is also thought that the cytosolic precursors of mitochondrial proteins have to maintain an unfolded conformation for membrane translocation (Hartl, *et al.*, 1989). This may be achieved by the interaction with the heat shock proteins of relative molecular mass 70 kDa and by the action of other factors in the cytosol

(Deshaies, *et al.*, 1988; Pelham, 1989). The transport of precursors across the two mitochondrial membranes are thought to occur in a single step at contact sites between the outer and inner membranes by a process requiring an electrochemical potential gradient across the inner mitochondrial membrane (Schleyer, *et al.*, 1982; Schleyer and Neupert, 1985; Schwaiger, *et al.*, 1987). The amino-terminal extensions are removed during, or shortly following, the translocation process by the action of a specific matrix localised metal dependent processing enzyme in a two step process (Pfanner, *et al.*, 1988; Schmidt, *et al.*, 1984; Hendrick, *et al.*, 1989).

The underlying biochemical anomaly leading to the storage of subunit *c* in lysosome derived lipopigment bodies remains to be defined. There are a number of possible mechanisms that could lead to its abnormal accumulation due to defect(s) occurring at several points in the normal biosynthetic pathway of this protein.

The gene sequences coding for subunit *c* (P1 and P2) in sheep affected with ovine ceroid-lipofuscinosis have been sequenced and shown to be identical to those of control sheep. Similar amounts of the mRNA for P1 and P2 were found in normal and diseased sheep liver tissue by Northern blot analysis (Medd, *et al.*, 1990). The storage of subunit *c* in lipopigment bodies is therefore not caused by a mutation in the presequences of either P1 or P2 resulting in misdirection of precursors of subunit *c* directly to lysosomes rather than to mitochondria, nor by a mutation affecting the normal gene products. These genes are normally regulated at the transcriptional level. Over-expression resulting in subunit *c* being synthesised in greater amounts than is normally required for assembly of the ATP synthase also seems unlikely on the basis of the Northern blot results. However, as the bovine P1 and P2 genes are known to be expressed in a tissue-specific manner (Gay and Walker, 1985), the possibility exists that the lesion in ovine ceroid-lipofuscinosis could result from a defect in the tissue-specific expression of these genes.

Little is known about the mechanisms underlying the folding and assembly of imported proteins into oligomeric enzyme complexes within

mitochondria nor about the coordination of assembly of mitochondrial and nuclear gene products. A nuclear encoded heat shock protein (hsp60) found in the mitochondrial matrix has recently been shown to be required for the correct assembly into oligomeric complexes of proteins imported into the mitochondrial matrix (Cheng, *et al.*, 1989; Reading, *et al.*, 1989). It has been suggested that a function of the hsp60 is to prevent the formation of misfolded proteins (Ostermann, *et al.*, 1989). The lesion could involve a defect in a similar component required for the correct folding and orientation of subunit *c* into the ATP synthase complex.

Subunit *c* plays a key role in the assembly and stability of the F_O-component, which is essential to the formation of a functional ATP synthase complex (Linnane, *et al.*, 1985; Hadikusumo, *et al.*, 1988). It is therefore unlikely that the genetic lesion involves a component required for correct assembly of subunit *c* into the ATP synthase oligomeric complex. Disruption of the ATP synthase would lead to uncoupling of oxidative phosporylation and subsequent death of the cell. Mitochondria isolated from diseased kidneys were also shown to maintain structural and functional integrity comparable to those isolated from control animals (Chapter 6).

Storage of the full subunit *c*, identical to that of the mature mitochondrial protein, suggests that the amino-terminal presequences have been cleaved by the specific protease located in the mitochondrial matrix. This implies a defect in its degradative pathway following correct incorporation into mitochondrial inner membranes. The normal amounts of this protein shown to be present in affected liver inner mitochondrial membranes by LDS-PAGE and the normal functioning of affected kidney mitochondria (Chapter 6) suggested that the accumulation was not occurring in the mitochondrion.

Lysosomes catabolise polymeric material by the action of many enzymes. There are a number of inborn errors of such catabolism affecting complex lipids, polysaccharides and mucopolyaccharides, but none have been described involving proteins. There is no reason why

such errors should not occur by chance. Failure to recognize them may be associated with the relative lack of specificity of lysosomal proteases. The cleavage specificities of lysosomal endopeptidases (cathepsins) and exopeptidases (Kirschke and Barrett, 1987) are such that a deficiency of one could be theoretically masked and covered by the activity of the remainder. Therefore a deficiency of a major cathepsin as a source of subunit *c* storage seems unlikely. However, there could be a specific unknown lysosomal protease required to degrade subunit *c* or more likely a cofactor specifically associated with the initial degradative step for this proteolipid. A deficiency of such a cofactor is known and accounts for one of the variants of GM_2 Gangliosidosis (Conzelmann and Sandhoff, 1978). It is also possible that the normal degradative enzymes associated with the catabolism of subunit *c* are unaffected and that subunit *c* is presented to them in a conformation that is resistant to proteolysis. Lipopigment storage bodies have been interpreted as complex paracrystalline structures that are not degraded by the lysosomal system (Jolly, *et al.*, 1989). Analogous protein inclusion body aggregates resistant to cellular proteases are found in bacteria as a result of over-expression of cloned mammalian genes (Cheng, *et al.*, 1981; Kane and Hartley, 1988)

Mitochondrial protein degradation and protein turnover are poorly understood. It is generally assumed that mitochondria are encapsulated into autophagic vacuoles and then degraded by a combination of lysosomal and mitochondrial proteases (Luzikov, 1985; Pfeifer, 1987). Lipopigment bodies in ovine foetuses and neonates have been described as whorls of bilayer membranes condensing into multilamellar paracrystalline structures (Jolly, *et al.*, 1989). These were thought to be the simplest and earliest lesions yet noted and suggested that autophagy of mitochondria was not the source of subunit *c* found in lipopigment bodies. The fact that no other mitochondrial ATP synthase protein components were shown to be stored during this study also suggest that this mechanism was unlikely to be the major route leading to accumulation of subunit *c* in lysosome derived lipopigment bodies.

Mitochondrial proteins have been shown to have a heterogeneity of

measured half lives ranging from 20 min to several days. Proteins within the same mitochondrial compartment and even within the same multisubunit complex have been shown to differ widely in their measured turnover rates (Lipskey and Pedersen, 1981; Hare and Hodges, 1982). These observations suggest the presence of mitochondrial proteolytic enzymes capable of selectively controlling the turnover of mitochondrial proteins. Several mitochondrial membrane bound proteases have been identified, including an ATP dependent, vanadate sensitive protease localised in the mitochondrial matrix which is capable of hydrolysing proteins to amino acids (Desautels and Goldberg, 1982a,b). An ATP independent protease that is capable of degrading oxidatively denatured proteins in the mitochondrial matrix has also been reported (Marcillat, *et al.*, 1988). It is not yet known whether any of these proteases are involved in the normal degradative pathway of subunit *c*. If the defect involves a mitochondrial protease then it is likely to involve an initial step in the catabolism of this molecule, as the full subunit *c* protein is stored.

Genetically distinct human and animal forms of the disease have been shown to store subunit *c*. This implies that they are likely to represent different mutations of the same or different genes associated with the metabolism of subunit *c*. There are many instances in other lysosomal storage diseases where a series of mutations affecting one or more genes results in storage of the same substrate e.g. GM_2 ganglioside (Neufeld, 1989). If two or more mutations affect the same gene, then intermediate diseases associated with double heterozygosity can be expected. Although this has not been demonstrated in any of the ceroid-lipofuscinoses, the large number of human variants suggest this possibility. Linkage studies have reported linkage of the juvenile form to the haptoglobin locus on chromosome 16 (Eiberg, *et al.*, 1989). Further linkage studies and gene mapping may help define the defective gene or genes. Inter breed genetic studies between clinically and pathologically distinct forms of the disease such as the Border Collie and the Tibetan Terriers, both shown to store subunit *c*, could indicate whether more than one gene is associated with defects of subunit *c* metabolism leading to ceroid-lipofuscinosis (Chapter 9).

Characterisation of the dominantly stored component in a storage disease should reflect the underlying metabolic defect. This characterisation has been achieved. Defining the metabolic process that leads to the abnormal accumulation of subunit *c* in lysosome derived lipopigment remains to be done. Its relationship to the neuronal necrosis may be a key to understanding the pathogenesis of the ceroid-lipofuscinoses. Storage of physically similar lipopigment occurs in a variety of other lysosomal storage diseases and with age. Severe neuronal necrosis is, however, unique to the ceroid-lipofuscinoses. This suggested that in the ceroid-lipofuscinoses, neuronal necrosis was associated with the metabolic lesion rather than storage of lipopigment *per se* (Jolly, *et al.* , 1989; 1990).

Research into the ceroid-lipofuscinoses is mainly driven by the need to understand their pathogeneses so that better diagnostic, control or therapeutic strategies can be developed. In light of the results presented in this study, elucidation of the biochemical defect may also help to further understand the biology of the mitochondrial ATP synthase enzyme complex.

REFERENCES

Anzil, A.P., Blinzinger, K., Harzer, K., Reither, M. and Zimmerman, G. (1975). Cytosome morphology and distribution of generalized ceroid-lipofuscinosis in a twenty-eight month old boy with normal myeloperoxidase activity. *Neuropadiatrie* **6** 259-283.

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

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

Armstrong, D., Dimmitt, S. and Van Wormer, D.E. (1974a). Studies in Batten disease. I. Peroxidase deficiency in granulocytes. *Arch. Neurol.* **30** 144-152.

Armstrong, D., Dimmitt, S., Boehme, D.H., Leonberg Jr., S.C. and Vogel, W. (1974b). Leucocyte peroxidase deficiency in a family with a dominant form of Kuf's disease. *Science* **186** 155-156.

Armstrong, D., Siakotos, A., Koppang, N. and Connele, E. (1978a). Studies on the retina and the pigment epithelium in hereditary canine ceroid lipofuscinosis. *Invest. Opthalmol. Vis. Sci.* **17** 608-617.

Armstrong, D., Connole, E., Feeney, L. and Berman, E. R. (1978b). Peroxidases in the neural retina and pigment epithelium. *J. Neurochem.* **31** 761-769.

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.

Armstrong, D. and Koppang, N. (1982). Histochemical evidence of lipid peroxidation in canine ceroid lipofuscinosis. In *Ceroid-lipofuscinosis (Batten's disease)* (Eds. Armstrong, D., Koppang, N. and Rider, J.A.). pp.159-165. Elsevier Biomedical Press, Amsterdam.

Awasthi, Y.C., Morris, H.H., Schochet Jr., S.S., Powell, G.F., Schmalstieg, F. C and Srivastava, S.K. (1977). Studies in neuronal ceroid-lipofuscinosis: Leukocyte peroxidase deficiency in a patient with neuronal ceroid-lipofuscinosis (Jansky-Bielschowsky type). *J. Lab. Clin. Med.* **89(4)** 770-780.

Ballard, F. J. (1982). Regulation of protein accumulation in cultured cells. *Biochem. J.* **208** 275-287.

Barber, M., Bordoli, R. S., Elliott, G. J., Sedgewick, R. D. and Tyler, A. N. (1983). Fast atom bombardment mass spectrometry (FABMS). A study of surface coverage effects in FABMS. *J. Chem. Soc., Faraday Trans. 1* **79** 1249-1255.

Becker, K., Goebel, H. -H., Svennerholm, L., Wendel, U. and Bremer, H. J. (1979). Clinical, morphological and biochemical investigations on a patient with an unusual form of ceroid-lipofuscinosis. *Eur. J. Pediatr.* **132** 197-206.

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.

Berkovic, S. F., Andermann, F., Andermann, E., Carpenter, S. and Wolfe, L. (1988). Kufs disease: Clinical features and forms. *Am. J. Med. Genet., Suppl.* **5** 105-109.

Biemann, K. (1982). Sequencing of proteins. *Int. J. Mass Spectrom. Ion. Phys.* **45** 183-194.

Bleistein, J., Heidrich, H. G. and Debuch, H. (1980). The phospholipids of liver lysosomes from untreated rats. *Hoppe-Seyler's Z. Physiol. Chem.* **351** 595-597.

Bolton, A. E. and Hunter, W. M. (1973). The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J.* **133** 529-539.

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.

Boustany, R-M. N. and Kolodny, E. H. (1989). Neurological progress. The neuronal ceroid lipofuscinoses: A review. *Rev. Neurol. (Paris).* **145(2)** 105-110.

Bowman, B. J., Allen, R., Wechser, M. A. and Bowman, E. J. (1988a). Isolation of genes encoding the *Neurospora* vacuolar ATPase. Analysis of *vma-2* encoding the 57-kDa polypeptide and comparison to *vma-1. J. Biol. Chem.* **263(28)** 14002-14007.

Bowman, E. J., Tenney, K. and Bowman, B. J. (1988b). Isolation of genes encoding the *Neurospora* vacuolar ATPase. Analysis of *vma-1* encoding the 67-kDa subunit reveals homology to other ATPases. *J. Biol. Chem.* **263(28)** 13994-14001.

Bozdech, M. J., Bainton, D. F. and Mustacchi, P. (1980). Partial peroxidase deficiency in neutrophils and eosinophils associated with neurologic disease. *Am. J. Clin. Path.* **73(3)** 409-416.

Butt, W. R. (1972). The iodination of follicle-stimulating and other hormones for radioimmunoassay. *J. Endocr.* **55** 453-454.

Cattell, K. J., Lindor, C. R., Knight, I. G. and Beechey, R. B. (1971). The identification of the site of action of NN'-Dicyclohexylcarbodi-imide as a proteolipid in mitochondrial membranes. *Biochem. J.* **125** 169-177.

Chang, S. E. (1986). In vitro transformation of human epithelial cells. *Biochim. Biophys. Acta* **823** 161-194.

Cheng, M. Y., Hartl, F-U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., Hallberg, E. M., Hallberg, R. L. and Horwich, A. L. (1989). Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. *Nature* **337** 620-625.

Cheng, Y-S., E., Kwoh, D. Y., Kwoh, T. J., Brook, 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. (Berl.)* **69** 161-164.

Clench, M. R., Garner, G. V., Gordon, D. B. and Barber, M. (1985). Surface effects in FAB mapping of proteins and peptides. *Biomed. Mass Spectrom.* **12(7)** 355-357.

Conzelmann, E. and Sandhoff, K. (1978). AB variant of infantile G_{M2}gangliosidosis: Deficiency of a factor necessary for stimulation of hexosaminidase A-catalyzed degradation of ganglioside G_{M2} and glycolipid G_{A2}. *Proc. Natl. Acad. Sci. USA* **75(8)** 3979-3983.

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

Daniel, P. F. (1990). Abnormal dolichol metabolism in NCL. Abstract in: *3rd International symposium on neuronal ceroid-lipofuscinoses. Indianapolis, 1990.*

Dawson, G. and Glaser, P. (1987). Apparent cathepsin B deficiency in neuronal ceroid lipofuscinosis can be explained by peroxide inhibition. *Biochem. Biophys. Res. Commun.* **147(1)** 267-274.

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

Dawson, G. (1990). Regulation of mammalian phospholipases and relevance to Batten disease. Abstract in: *3rd International symposium on neuronal ceroid-lipofuscinosis. Indianapolis, 1990.*

Den Tandt, W.R. and Martin, J.J. (1978). Peroxidase in ceroid-lipofuscinosis. *J. Neurol. Sci.* **38** 191-193.

Dermietzel, R., Volker, M., Hwang, T-K., Berzborn, R. J. and Meyer, H. E. (1989). A 16 kDa protein co-isolating with gap junctions from brain tissue belonging to the class of proteolipids of the vacuolar H^+-ATPases. *FEBS Lett.* **253(1&2)** 1-5.

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

Desautels, M. and Goldberg, A. L. (1982b). Demonstration of an ATP-dependent, vanadate-sensitive endoprotease in the matrix of rat liver mitochondria. *J. Biol. Chem.* **257(19)** 11673-11679.

Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A. and Schekman, R. (1988). A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature* **332** 800-810.

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

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. (1990). Sequence analysis of members of the human gene family for the proteolipid subunit of mitochondrial ATP synthase. *J. Mol. Biol.* (In Press).

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

Dyrks, T., Weidemann, A., Multhaup, G., Salbaum, J. M., Lemaire, H-G., Kang, J., Muller-Hill, B., Masters, C. L. and Beyreuther, K. (1988). Identification, transmembrane orientation and biogenesis of the amyloid A4 precursor of Alzheimer's disease. *EMBO J.* **7(4)** 949-957.

Edman, P. and Begg, G. (1967). A protein sequenator. *Eur. J. Biochem.* **1** 80-91.

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

Elleder, M. (1981). Chemical characterization of age pigments. In *Age Pigments* (Ed. Sohal, R.S.). pp.203-241. Elsevier North-Holland Biomedical Press, Amsterdam.

Estabrook, R. W. (1967). Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. In *Methods in Enzymology Volume X Oxidation and phosphorylation* (Eds. Estabrook, R. W. and Pullman, M. E.). pp. 41-47. Academic Press, New York.

Eto, Y., Tsuda, T., Harada, E., Ohashi, T. and Tokoro, T. (1990). The role of proteases and lipids in neuronal ceroid lipofuscinosis (NCL). Abstract in: *3rd International symposium on neuronal ceroid-lipofuscinosis. Indianapolis, 1990.*

Farnsworth, C., Armstrong, D., Koppang, N., Handelman, G., Stone, W. and Dratz, E. (1982). Canine ceroid lipofuscinosis: Plasma fatty acid composition and glutathione peroxidase activity as a function of age. In *Ceroid-lipofuscinosis (Batten's disease)* (Eds. Armstrong, D., Koppang, N. and Rider, J.A.). pp.271-282. Elsevier Biomedical Press, Amsterdam.

Farrell, L. B., Gearing, D. P. and Nagley, P. (1988). Reprogrammed expression of subunit 9 of the mitochondrial ATPase complex of *Saccharomyces cerevisiae*. Expression *in vitro* from a chemically synthesized gene and import into isolated mitochondria. *Eur. J. Biochem.* **173** 131-137.

Fearnley, I. M. and Walker J. E. (1986). Two overlapping genes in bovine mitochondrial DNA encode membrane components of ATP synthase. *EMBO J.* **5(8)** 2003-2008.

Fillingame, R. H. (1976). Purification of the carbodiimide-reactive protein component of the ATP energy-transducing system of *Escherichia coli*. *J. Biol. Chem.* **251(21)** 6630-6637.

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

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

Folch, J., Lees, M. and Sloane Stanley, G.H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226** 497-509.

Friedman, R.D. (1982). Comparison of four different silver-staining techniques for salivary protein detection in alkaline polyacrylamide gels. *Anal. Biochem.* **126** 346-349.

Gadoth, N. (1978). Neuronal ceroid-lipofuscinosis (Batten's disease): Diagnostic approach and results of a therapeutic trial. *Metab. Opthalmol.* **2** 193-196.

Garrod, A. E. (1908). In-born errors of metabolism. *(Croonian lectures) Lancet 2, i 73, 142, 214.* cited by Stanbury *et al.* (1978). In *The Metabolic Basis Of Inherited Disease.* (Eds. Stanbury, J. B., Wyngaarden, J. B. and Fredrickson, D. S.). pp. 2-31. McGraw-Hill Book Co., New York.

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(13A)** 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 Suppl. 2* 329-332.

Goebel, H. H., Kohnecke, B. and Koppang, N. (1982). Ultrastructural studies on the retina in human and canine neuronal ceroid-lipofuscinoses and other lysosomal disorders. *Birth Defects: Original Article Series* **18(6)** 241-253.

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

Graf, T. and Sebald, W. (1978). The dicyclohexylcarbodiimide-binding protein of the mitochondrial ATPase complex from beef heart. Isolation and amino acid composition. *FEBS Lett.* **94(2)** 218-222.

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. Opthalmol. 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.

Greenwood, F. C. and Hunter, W. M. (1963). The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89** 114-123.

Gutteridge, J. M. C., Heys, A. D. and Lunec, J. (1977). Fluorescent malondialdehyde polymers from hydrolysed 1, 1, 3, 3,-tetramethoxypropane. *Anal. Chim. Acta* **94** 209-211.

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

Gutteridge, J.M.C., Westermarck, T. and Santavuori, P. (1983). Iron and oxygen radicals in tissue damage: implications for the neuronal ceroid lipofusinoses. *Acta Neurol. Scand.* **68** 365-370.

Hadikusumo, R. G., Meltzer, S., Choo, W. M., Jean-Francois, M. J. B., Linnane, A. W. and Marzuki, S. (1988). The definition of mitochondrial H^+ ATPase assembly defects in *mit* $^-$ mutants of *Saccharomyces cerevisiae* with monoclonal antibody to the enzyme complex as an assembly probe. *Biochim. Biophys. Acta* **933** 212-222.

Hagberg, B., Sourander, P. and Svennerholm, L. (1968). Late infantile progressive encephalopathy with disturbed polyunsaturated fat metabolism. *Acta Paediat. Scand.* **57** 495-499.

Hagberg, B., Haltia, M., Sourander, P., Svennerholm, L. and Eeg-Olufsson, O. (1974). Polyunsaturated fatty acid lipidosis. Infantile form of so-called neuronal ceroid lipofuscinosis. I. Clinical and morphological aspects. *Acta Paediatr. Scand.* **63** 753-763.

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. *Am. J. Med. Genet.* (In Press).

Hare, J. F. and Hodges, R. (1982). Turnover of mitochondrial inner membrane proteins in hepatoma monolayer cultures. *J. Biol. Chem.* **257(7)** 3575-3580.

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. (Berl.)* **75** 632-636.

Hartl, F-U., Schmidt, B., Wachter, E., Weiss, H. and Neupert, W. (1986). Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of ubiquinol-cytochrome c reductase. *Cell* **47** 939-951.

Hartl, F-U., Ostermann, J., Guiard, B. and Neupert, W. (1987). Successive translocation into and out of the mitochondrial matrix: Targeting of proteins to the intermembrane space by a bipartite signal peptide. *Cell* **51** 1027-1037.

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

Haust, M.D., Gordon, B.A. and Hinton, G.G. (1976). Ultrastructure and peroxidase of leucocytes in five patients with juvenile form of ceroid lipofuscinosis. In *Current Trends in Sphingolipidoses and Allied Disorders* (Eds. Volk, B.W. and Schneck, L.). pp.413-427. Plenum Press, New York.

Heiskala, H., Gutteridge, J. M. C., Westermarck, T., Alanen, T. and Santavuori, P. (1988). Bleomycin-detectable iron and phenanthroline-detectable copper in the cerebrospinal fluid of patients with neuronal ceroid-lipofuscinoses. *Am. J. Med. Genet., Suppl.* **5** 193-202.

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.

Hirs, C. H. W. (1967). Determination of cystine as cysteic acid. In *Methods in Enzymology Volume XI Enzyme Structure* (Ed. Hirs, C. H. W.). pp. 59-62. Academic Press, New York.

Hubbard, A. L. and Cohn, Z. A. (1972). The enzymatic iodination of the red cell membrane. *J. Cell. Biol.* **55** 390-405.

Ivy, G.O., Schottler, F., Wenzel, J., Baudry, M. and Lynch, G. (1984). Inhibitors of lysosomal enzymes: Accumulation of lipofuscin-like dense bodies in the brain. *Science* **226** 985-987.

Jensen, G.E., Clausen, J., Melchior, J.C. and Konat, G. (1977). Clinical, social and biochemical studies on Battens syndrome, alias Spielmeyer-Vogt or Stengel syndrome. *Eur. Neurol.* **15** 203-211.

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.

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. and Palmer, D. N. (1990). The ovine and other models of ceroid-lipofuscinosis: Their relevance to Batten's disease. *Am. J. Med. Genet.* (In Press).

Joshi, U. M., Rao, P., Kodavanti, 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 in inclusion bodies in *Escherichia coli TIBTEC* **6** 95-101.

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.

Keller, R.K., Armstrong, D., Crum, F.C. and Koppang, N. (1984). Dolichol and dolichyl phosphate levels in brain tissue from English setters with ceroid lipofuscinosis. *J. Neurochem.* **42** 1040-1047.

Kirschke, H. and Barrett, A. J. (1987). Chemistry of lysosomal proteases. In *Lysosomes Their Role In Protein Breakdown* (Eds. Glaumann, H. and Ballard, F. J.). pp. 193-238. Academic Press, London.

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.

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

Koppang, N. (1970). Neuronal ceroid-lipofuscinosis 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. (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.

Kratzin, H. D., Wiltfang, J., Karas, M., Neuhoff. V. and Hilschmann, N. (1989). Gas-phase sequencing after electroblotting polyvinylidene difluoride membranes assigns correct molecular weights to myoglobin molecular weight markers. *Anal. Biochem.* **183** 1-8.

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

Lake, B. D. (1984). Lysosomal enzyme deficiencies. In *Greenfields Neuropathology.* (Eds. Adams, J. H., Corsellis, J. A. W., Duchen, L. W.). pp. 491-572. Edward Arnold, London.

Lees, M. B., Sakura, J. D., Sapirstein, V. S. and Curatolo, W. (1979). Structure and function of proteolipids in myelin and non-myelin membranes. *Biochim. Biophys. Acta.* **559** 209-230.

Linnane, A. W., Lukins, H. B., Nagley, P., Marzuki, S., Hadikusumo, R. G., Jean-Francois, M. J. B., John, U. P., Ooi, B. G., Watkins, L., Willson, T. A., Wright, J. and Meltzer, S. (1985). Assembly of the yeast mitochondrial H^+-ATPase: Correlative studies involving gene sequencing and immunochemical probes of assembly. In *Achievements and Perspectives of Mitochondrial Research Volume 1: Bioenergetics* (Eds. Quagliariello, E., Slater, E. C., Palmieri, F., Saccone, C. and Kroon, A. M.). pp. 211-222. Elsevier/North-Holland Biomedical Press, Amsterdam.

Lipskey, 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(16)** 8652-8657.

Luzikov, V. N. (1985). Chapter four: Mitochondrial degradation in the cell. In *Mitochondrial Biogenesis And Breakdown* (Ed. Luzikov, V. N.). pp.132-153. Consultants Bureau, New York.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193** 265-275.

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.

Manolson, M. F., Ouellette, B. F. F., Filion, M. and Poole, R. J. (1988). cDNA sequence and homologies of the "57-kDa" nucleotide-binding subunit of the vacuolar ATPase from Arabidopsis. *J. Biol. Chem.* **263(34)** 17987-17994.

Marchalonis, J. J. (1969). An enzymic method for the trace iodination of immunoglobulins and other proteins. *Biochem. J.* **113** 299-305.

Marcillat, O., Zhang, Y., Lin, S. W. and Davies, K. J. A. (1988). Mitochondria contain a proteolytic system which can recognize and degrade oxidatively-denatured proteins. *Biochem. J.* **254** 677-683.

Markwell, M. A. K. and Fox, C. F. (1978). Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril. *Biochemistry* **17(22)** 4807-4817.

Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262(21)** 10035-10038.

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.

Medd, S. M., Walker, J. E., Fearnley, I. M., Jolly, R. D. and Palmer, D. N. (1990). Lysosomal storage of a mitochondrial protein in Batten's disease (Ceroid lipofuscinosis). In *Molecular basis of neurological disorders and their treatment* (In Press).

Merril, C. R. (1986). Development and mechanisms of silver stains for electrophoresis. *Acta Histochem. Cytochem.* **19(5)** 655-667.

Moriyama, Y. and Nelson, N. (1989). Lysosomal H^+-translocating ATPase has a similar subunit structure to chromaffin granule H^+-ATPase complex. *Biochim. Biophys. Acta* **980** 241-247.

Nedergaard, J. and Cannon, B. (1979). Overview-Preparation and properties of mitochondria from different sources. In *Methods in Enzymology volume LV Biomembranes Part F: Bioenergetics-oxidative phosphorylation* (Eds. Fleischer, S. and Packer, L.). pp. 3-9. Academic Press, New York.

Nelson, E.C. and Halley, B.A. (1977). Retinoyl complexes in Batten disease. *Science* **198** 527-528.

Neufeld, E. F. (1989). Natural history and inherited disorders of a lysosomal enzyme, β-Hexosaminidase. *J. Biol. Chem.* **264(19)** 10927-10930.

Ng Ying Kin, N.M.K. and Wolfe, L.S. (1982). Presence of abnormal amounts of dolichols in the urinary sediment of Battens disease patients. *Pediatr. Res.* **16** 530-532.

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(5)** 1465-1473.

Ostermann, J., Horwich, A. L., Neupert, W. and Hartl, F-U. (1989). Protein folding in mitochondria requires complex formation with hsp60 and ATP hydrolysis. *Nature* **341** 125-130.

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(4)** 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(4)** 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., 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. 221-233. Plenum Press, New York.

Patel, V., Koppang, N., Patel, B. and Zeman, W. (1974). p - Phenylenediamine-mediated peroxidase deficiency in English Setters with neuronal ceroid-lipofuscinosis. *Lab. Invest.* **30** 366-368.

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.

Pearse, A. G. E. (1985). *Histochemistry, Theoretical and Applied. Vol 2. Analytical Technology. 4th Edition* pp. 686-690. Churchill Livingston, Edinburgh.

Pelham, H. R. B. (1989). Heat shock and the sorting of luminal ER proteins. *EMBO J.* **8(11)** 3171-3176.

Pfaller, R. and Neupert, W. (1987). High-affinity binding sites involved in the import of porin into mitochondria. *EMBO J.* **6(9)** 2635-2642.

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(6)** 2483-2490.

Pfanner, N., Hartl, F-U. and Neupert, W. (1988). Import of proteins into mitochondria: a multi-step process. *Eur. J. Biochem.* **175** 205-212.

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.

Pilz, H., O'Brien, J.S. and Heipertz, R. (1976a). Human leucocyte peroxidase: Activity of a soluble and membrane-bound enzyme form in normal persons and patients with neuronal ceroid-lipofuscinosis. *Metabolism* **25** 561-570.

Pilz, H., O'Brien, J.S. and Heipertz, R. (1976b). Human saliva peroxidase: Microanalytical isoelectric fractionation and properties in normal persons and in cases with neuronal ceroid-lipofuscinosis. *Clin. Biochem.* **9** 85-88.

Pilz, H. and Goebel, H.H. (1977). Peroxidase activity in the parotid gland in juvenile neuronal ceroid-lipofuscinosis. *Neuropathol. Appl. Neurobiol.* **3** 93-101.

Pongor, S., Ulrich, P. C., Bencsath, F. A. and Cerami, A. (1984). Aging of proteins: Isolation and identification of a fluorescent chromophore from the reaction of polypeptides with glucose. *Proc. Natl. Acad. Sci. USA* **81** 2684-2688.

Porta, E. A. and Hartroff, W.S. (1969). Lipid pigments in relation to aging and dietary factors (lipofuscins). In *Pigments in Pathology* (Ed. Wolfan, M.). pp. 191-235. Academic Press, New York.

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.

Pullarkat, R.K. and Raguthu, S. (1985). Elevated urinary dolichol levels in chronic alcoholics. *Alchol. Clin. Exp. Res.* **9** 28-30.

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: *3rd International symposium on neuronal ceroid-lipofuscinoses. Indianapolis, 1990.*

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

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

Reading, D. S., Hallberg, R. L. and Myers, A. M. (1989). Characterization of the yeast *HSP60* gene coding for a mitochondrial assembly factor. *Nature* **337** 655-659.

Reddy, T. S., Armstrong, D. and Bazan, N. G. (1985). Arachidonic acid and other long-chain fatty acids in canine ceroid lipofuscinosis. Distribution in glycerolipids, metabolism, and pathophysiological correlations. *Neurochem. Pathol.* **3** 83-97.

Redshaw, M. R. and Lynch, S. S. (1974). An improved method for the preparation of iodinated antigens for radioimmunoassay. *J. Endocr.* **60** 527-528.

Reid, J.C.W. and Husbands, D.R. (1985). Oxidative metabolism of long-chain fatty acids in mitochondria from sheep and rat liver. Evidence that sheep conserve linoleate by limiting its oxidation. *Biochem. J.* **225** 233-237.

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., Cummings, J. F., Loew, E. and deLahunta, A. (1990). The Tibetan Terrier and Kuffs disease. *Am. J. Med. Genet.* (In Press).

Salacinski, P. R. P., McLean, C., Sykes, J. E. C., Clement-Jones, V. V. and Lowry, P. J. (1981). Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidising agent, 1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril (Iodogen). *Anal. Biochem.* **117** 136-146

Santavuori, P. and Westermarck, T. (1984). Antioxidant therapy in neuronal ceroid-lipofuscinosis. *Med. Biol.* **62** 152-153.

Santavuori, P., Heiskala, H., Westermarck, T., Sainio, K. and Moren, R. (1988). Experience over 17 years with antioxidant treatment in Spielmeyer-Sjogren disease. *Am. J. Med. Genet., Suppl.* **5** 265-274.

Schleyer, M., Schmidt, B. and Neupert, W. (1982). Requirement of a membrane potential for the posttranslational transfer of proteins into mitochondria. *Eur. J. Biochem.* **125** 109-116.

Schleyer, M. and Neupert, W. (1985). Transport of proteins into mitochondria: Translocation intermediates spanning contact sites between outer and inner membranes. *Cell* **43** 339-350.

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.* **114** 581-588.

Schwaiger, M., Herzog, V. and Neupert, W. (1987). Characterization of translocation contact sites involved in the import of mitochondrial proteins. *J. Cell Biol.* **105** 235-246.

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.

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. Opthalmol. Vis. Sci.* **17(7)** 618-633.

Sierra, M. F. and Tzagoloff, A. (1973). Assembly of the mitochondrial membrane system. Purification of a mitochondrial product of the ATPase. *Proc. Natl. Acad. Sci. USA* **70(11)** 3155-3159.

Srivastava, S. K., Garg, H. S. and Awasthi, Y. C. (1982). Quantitaion of ceroid and lipofuscin pigments in the brain tissue of normal subjects and a patient with Batten-Spielmeyer-Vogt syndrome. In *Ceroid-lipofuscinosis (Batten's disease)* (Eds. Armstrong, D., Koppang, N. and Rider, J. A.). pp.185-188. Elsevier Biomedical Press, Amsterdam.

Stampfer, M. R. and Bartley, J. C. (1985). Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[α]pyrene. *Proc. Natl. Acad. Sci. USA* **82** 2394-2398.

Svennerholm, L., Hagberg, B., Haltia, M., Sourander, 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.

Svennerholm, L., Fredman, P., Jungbjer, B., Mansson, J-E., Rynmark, B-M., Bostrom, K., Hagberg, B., Noren, L. and Santavuori, P. (1987). Large alterations in ganglioside and neutral glycosphingolipid patterns in brains from cases with infantile neuronal ceroid lipofuscinosis/polyunsaturated fatty acid lipidosis. *J. Neurochem.* **49(6)** 1772-1783.

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

Tsan, M-F., Gale, A.N., Murphy, E.A. and McIntrye, P.A. (1978). Neuronal ceroid-lipofuscinosis. Studies of granulocyte enzyme activities. *J. Neurol. Sci.* **36** 13-24.

Tzagoloff, A. (1982). Chapter 6: Oxidative phosphorylation. In *Mitochondria* (Ed. Tzagoloff, A.). pp.1-156. Plenum Press, New York.

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

van Loon, A. P. G. M., Brandli, A. W., Pesold-Hurt, B., Blank, D. and Schatz, G. (1987). Transport of proteins to the mitochondrial intermembrane space: the 'matrix-targeting' and the 'sorting' domains in the cytochrome c_1 presequence. *EMBO J.* **6(8)** 2433-2439.

van Loon, A. P. G. M. and Schatz, G. (1987). Transport of proteins to the mitochondrial intermembrane space: the 'sorting' domain of the cytochrome c_1 presequence is a stop-transfer sequence specific for the mitochondrial inner membrane. *EMBO J.* **6(8)** 2441-2448.

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

Walker, J. E., Fearnley, I. M., Lutter, R., Todd, R. J. and Runswick, M. J. (1990). Structural aspects of proton-pumping ATPases. *Phil. Trans. R. Soc. Lond. B.* **326** 367-378.

Weast, R. C., Astle, M. J. and Beyer, W. H. (Eds.). (1988). *CRC Handbook of Chemistry and Physics, 69th Edition.* pp. D225-D226. CRC Press, Inc. Boca Raton, Florida.

Wilkie, J. S. N. and Hudson, E. B. (1982). Neuronal and generalized ceroid-lipofuscinosis in a Cocker Spaniel. *Vet. Pathol.* **19** 623-628.

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.

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., Palo, J. and Haltia, M. (1982). Raised levels of cerebral cortex dolichols in Alzheimer's disease. *The Lancet* 99.

Wolfe, L. S., Palo, J., Santavuori, P., Andermann, F., Andermann, E., Jacob, J. C. and Kolodny, E. (1986). Urinary sediment dolichols in the diagnosis of neuronal ceroid-lipofuscinosis. *Ann. Neurol.* **19(13)** 270-274.

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. (1988). Dolichol and dolichyl phosphate in the neuronal ceroid-lipofuscinoses and other diseases. *Am. J. Med. Genet., Suppl.* **5** 233-242.

Wong, T. K., Decker, G. L. and Lennarz, W. J. (1982). Localization of dolichol in the lysosomal fraction of rat liver. *J. Biol. Chem.* **257(11)** 6614-6618.

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.

Zeman, W. and Dyken, P. (1969). Neuronal ceroid-lipofuscinosis (Batten's disease): Relationship to amaurotic family idiocy? *Pediatrics* **44(4)** 570-583.

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

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

Zimniak, L., Dittrich, P., Gogarten, J. P., Kibak, H. and Taiz, L. (1988). The cDNA sequence of the 69-kDa subunit of the carrot vacuolar H^+-ATPase. Homology to the β-chain of F_0F_1-ATPases. *J. Biol. Chem.* **263(19)** 9102-9112.

Zwizinski, C., Schleyer, M. and Neupert, W. (1984). Proteinaceous receptors for the import of mitochondrial precursor proteins. *J. Biol. Chem.* **259(12)** 7850-7856.