BIOCHEMICAL STUDIES ON OVINE CEROID-LIPOFUSCINOSIS

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ABSTRACT

The ceroid-lipofuscinoses are a group of inherited diseases of humans and animals characterised by brain atrophy and the storage of a fluorescent lipopigment in a variety of tissues. Clinical signs include loss of vision, seizures, mental retardation and dementia, with the disease culminating in premature death. Defects in lipid metabolism or the control of lipid peroxidation have been postulated to explain their pathogenesis. Specific defects in peroxidases, fatty acid metabolism, dolichol metabolism, retinol metabolism and iron metabolism have been proposed. Evidence for these mechanisms arises from the apparent lipoid fluorescent Schiff base nature of the lipopigment.

This study reports the analysis of total tissue lipids and lipopigment isolated from tissues of sheep affected with ceroid-lipofuscinosis. Brain grey matter phospholipid fatty acids of diseased sheep were compared with those of normal sheep. Phosphatidylethanolamine of diseased sheep contained more 18:1(n-9) and less 22:6(n-3) than normal and their phosphatidylcholine less 16:0. Other differences were minor. No differences were found between the liver lipids nor the fatty acid profiles of their phosphatidylcholine, phosphatidylethanolamine or triglycerides.

Lipopigment from the liver of affected sheep was 70% proteinaceous, the rest being mainly lipids. These were only one sixth as fluorescent as total liver lipids, but contained a number of fluorophors. None were major components of the lipopigment or the postulated fluorescent product of lipid peroxidation. Lipopigment lipids included the lysosomal marker bis(monoacylglyceryl)phosphate that contained 42.9% linoleate and 16.5% linolenate. Lipopigment neutral lipids were dolichol, dolichyl esters, ubiquinone, free fatty acids and cholesterol, indicative of a lysosomal origin of the lipopigment. Phosphatidylcholine, phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine were present in proportions and with fatty acid profiles typical of lysosomes.

Lipopigment isolated from liver, kidney, pancreas and brain of affected sheep without the use of proteolytic enzymes was two-thirds protein. Silver staining after sodium dodecyl sulphate polyacrylamide gel electrophoresis showed a major band of M_r 14,800, heterogeneous material between 5,000 - 9,000 M_r and a major band of M_r<3,500. These components did not stain for RNA or carbohydrate, and were digested by a nuclease free protease. They are not normal lysosomal proteins. The presence of the 3,500 M_r proteins in whole affected tissue homogenates distinguished them from homogenates of normal tissue. Lipopigment levels of dolichol, ubiquinone and cholesterol were consistent with the lipopigment being protein enriched lysosome derived cytosomes.

The concentration of metals in lipopigment from the four tissues was also analysed. Liver lipopigment had a high copper content, 1.3%, kidney lipopigment a high iron content and brain lipopigment showed an accumulation of some trace elements. These data indicate that the lipopigment cytosomes have a history of being functional lysosomes as far as metal metabolism is concerned. Metal analyses of cerebrospinal fluid revealed no indication of a defect in metal metabolism.

It is concluded that ovine ceroid-lipofuscinosis is not a lipidosis, nor does the lipopigment arise from the abnormal peroxidation of lipids. There is no evidence of any disturbance in metal metabolism. Low molecular weight proteins are stored in lysosome derived organelles and on these grounds the ceroid-lipofuscinoses should be regarded as lysosomal proteinoses. These may result from defects in lysosomal proteolysis or its control.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

THE BIOCHEMISTRY OF CEROID-LIPOFUSCINOSIS : A REVIEW

Introduction

The neuronal ceroid-lipofuscinoses are a group of storage diseases of children inherited as autosomal recessive traits. Children appear to develop normally at first but clinical symptoms develop in infancy or in childhood. Clinical signs include loss of vision, seizures, mental retardation and dementia, with the disease culminating in premature death. Three main types occur, namely the infantile, late infantile and juvenile forms, distinguished from each other by the age of onset and progress of clinical disease. Sub-types also occur (Zeman, 1976; Lake, 1977; Lake and Cavanagh, 1978). The close homochronism and homotypism between affected siblings (Zeman, 1976) indicates that the subdivision into groups is genetically determined. An adult form, Kufs disease, has been described.

The first clinical description of what is now called ceroid-lipofuscinosis was made in 1826 (Stengel, 1826). For over one hundred years these diseases were grouped with the other amaurotic familial idocies. In 1969 they were delineated from the gangliosidoses in an extensive pathological study (Zeman and Dyken, 1969; Zeman, 1970; Zeman et al., 1970), a possibility previewed by Spielmeyer in 1906 (Spielmeyer, 1906). They were collectively renamed the "ceroid-lipofuscinoses" because of the similarities of the lipopigment abnormally stored in cells of affected people to the lipopigments ceroid and lipofuscin. These diseases have also been known by a large number of eponyms (Table I) of which Batten's disease is the best known. Eponyms have sometimes been used alone, e.g. Batten disease, or in combination, e.g. Spielmeyer-Vogt syndrome.

Table 1

Nomenclature of the ceroid-lipofuscinoses.

Descriptive	Eponyms
Infantile	Haltia-Santavuori
Late Infantile	Jansky-Bielschowsky
Juvenile	Batten-Mayou-Spielmeyer Vogt-Sjogren
Adult	Kuf

The ceroid-lipofuscinoses are not restricted to any particular population. They are probably the most common type of inherited storage disease, affecting approximately 1 in 100,000 live births world-wide (Zeman et al. 1970). Diagnosed cases however are more common in Scandinavia (Gutteridge et al., 1982a) but this may in part be a consequence of better diagnostic methods there (Norio, et al., 1973). The prevalence of the infantile form in Finland is approximately 1 in 13,000 (Santavuori et al., 1974). Similar diseases have been found in domestic animals, namely in Beefmaster cattle (Read and Bridges, 1969), Siamese cats (Green and Little, 1974), Chihuahua dogs (Rac and Giesecke, 1975), Dachshunds (Cummings and de Lahunta, 1977), Saluki dogs (Appleby et al., 1984), Dalmatian dogs (Goebel and Dahme, 1985), Blue Heeler dogs (Cho et al., 1986), a mature cross-bred terrier (Hoover et al., 1984), English Setter dogs (Koppang, 1970) and New Zealand South Hampshire sheep (Jolly and West, 1976). The diseases in the English Setters and South Hampshire sheep have been studied as models of the human diseases and biochemical studies of affected South Hampshire sheep form the basis of this thesis. A flock has been bred and maintained for this purpose. These sheep have been the subject of extensive pathological studies which have established them as a good model, most resembling the juvenile form of the human disease (Jolly et al. 1980, 1982; Graydon and Jolly, 1984; Mayhew et al. 1985).

The ceroid-lipofuscinoses are characterised by severe brain atrophy, a lesion unique among the inherited storage diseases. Similar secondary disease is not seen in other organs even though pigment cytosomes are present in most cell types of the body. The cytosomes are osmiophilic, fluorescent, periodic acid Schiff (PAS) and Sudan stain positive bodies. Their general staining and fluorescent properties have led them to be deemed lipopigment. Ultrastructural studies have shown them to be cytosomes consisting of dense granular areas and a variety of multilayered membranous arrays. The reason for this diversity of structure is not understood. Attempts to classify the sub-types of the disease in terms of the relative proportions of these morphologically varied structures have met with only limited success (Goebel et al., 1979). Studies on affected sheep have shown that different tissues in the same affected animal contain cytosomes of varied appearance (Jolly et al., 1980).

Although clinical disease is not manifested for several months or years, lipopigment is probably present at all ages. It occurred in amniotic cells of a 16 week old foetus, an observation that allowed prenatal diagnosis by electron microscopy (McCleod, et al., 1984). It is also present in brains of affected lambs in quantities sufficient to allow diagnosis by histopathology of paraffin blocked sections at birth (Jolly pers. comm.).

Lipopigments and their terminology

Lipopigments are a family of fluorescent cytosomes that stain with lipid stains in histological preparations. Generally they are coloured yellow to brown, but sometimes they are almost colourless. Their biogenesis is not always clear and it is probable that they result from a variety of different causes. The term lipofuscin, derived from "lipo" meaning fat (Greek), and "fuscin" meaning dusky brown (Latin), was introduced by Borst (1922). Initially its use was reserved for "age-pigment", i.e. the lipopigment that accumulates with age, particularly in post-mitotic cells, but "lipofuscin" is now sometimes used to describe other similar lipopigments that can occur for a variety of reasons, not all of which are understood. A lysosomal origin of lipofuscin is generally accepted.

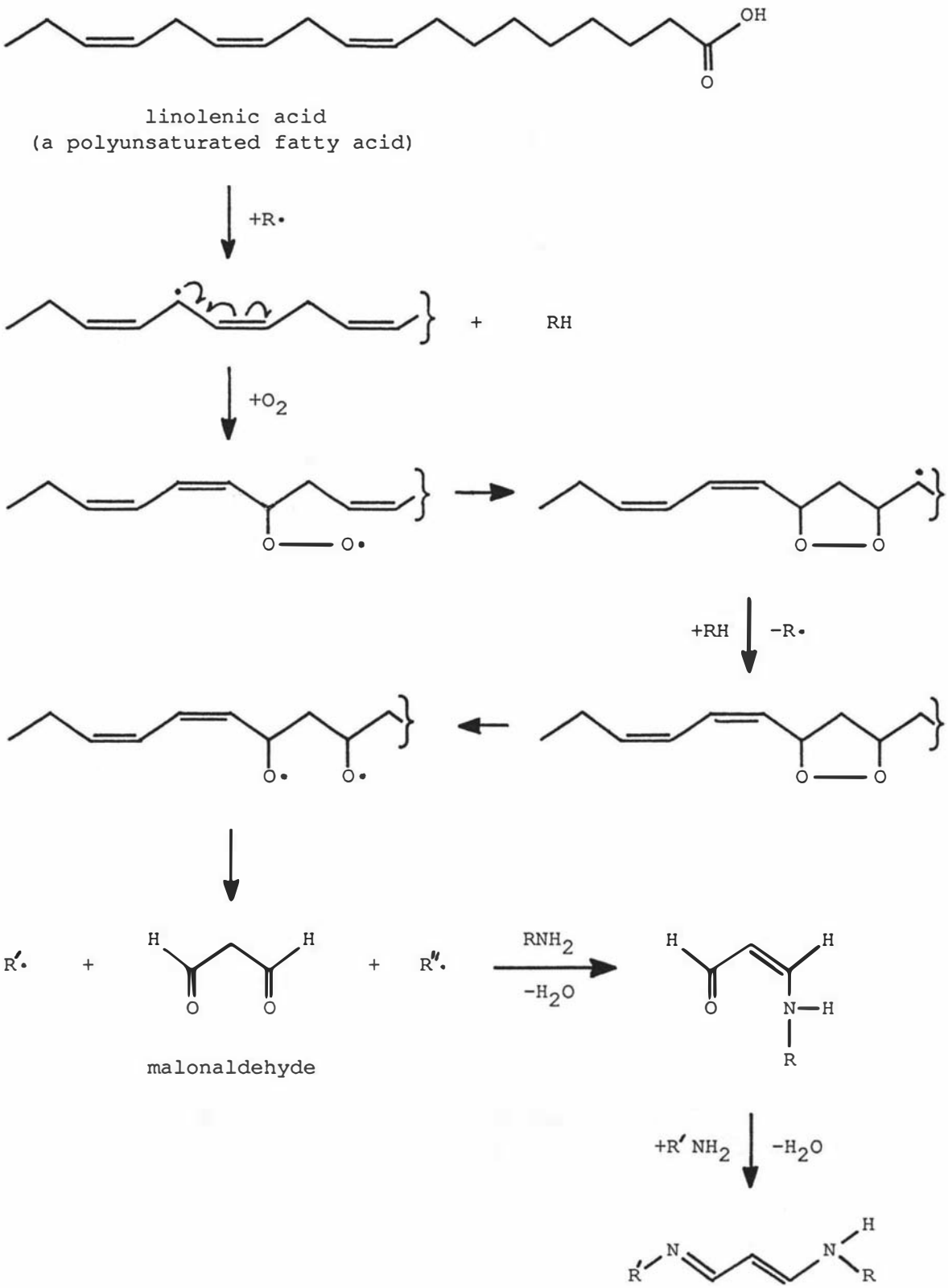
Ceroid generally refers to a lipopigment which results from the degeneration of lipid associated with fat necrosis or the storage of lipid in unusual amounts. It is usually found within macrophages (Schroder, 1980). The term ceroid has also been used for any lipopigment whose presence is indicative of a disease state.

Some authors apply both these terms ceroid and lipofuscin, to the entire cytosomal structure while others implicate only part of it, usually the so-called fluorophor. The terms lipofuscin and ceroid will be used in this thesis only within the above more classical definitions. Other lipopigments will be referred to as lipopigments and their origin indicated. Lipopigment, age-pigment, lipofuscin and ceroid will all refer to the entire cytosomes, rather than any part thereof.

Proposed mechanisms of lipopigment formation

Efforts to understand the biogenesis of the various lipopigments and of the lipopigment in the ceroid-lipofuscinoses have concentrated on possible mechanisms to explain their fluorescence. A degree of commonality as to the origin of all these structurally similar lipopigments has been assumed. In 1969 Chio and Tappel reported that material with the fluorescent properties of lipofuscin was generated when protein reacted with peroxidised lipids, and furthermore that Schiff bases with the same fluorescent properties were generated when malonaldehyde was reacted with a number of amino acids (Chio and Tappel, 1969 a, b). As malonaldehyde is a product of lipid peroxidation, the following schema emerged (Fig.1) with the generated malonaldehyde reacting with the amino groups of proteins or phospholipids to form the fluorophor. Because lipofuscin has similar fluorescent spectra, an analogous fluorophor was postulated as its core. Consequently by the same analogy a similar fluorophor was postulated as the primary element in the fluorescent cytosomes of the ceroid-lipofuscinoses.

Fig.1. Proposed mechanism of polymeric Schiff base formation.



In part from T.F. Slater (1972)
"Free Radical Mechanism in Tissue Injury"
p.31, PION Press, London.

N,N -disubstituted
1-amino-3-iminopropene
(a Schiff base)

The apparent lipid nature of the stored lipopigment had already drawn attention to the possible involvement of abnormal lipid metabolism in the pathogenesis of the diseases. Early biochemical studies had concentrated on the sphingolipids, i.e. cerebrosides and gangliosides, as these compounds were implicated in the other forms of amaurotic idiocy e.g. the gangliosidoses. No useful information emerged from these early studies and the work was abandoned. The development of an hypothesis that postulated a defect in the metabolism of a different class of lipid, i.e. polyunsaturated fats, although not entirely novel, was consequently greeted with alacrity (Zeman and Dyken, 1969; Zeman, 1974; Zeman, 1976). Four main lines of supporting evidence quickly emerged, namely disturbances in fatty acid profiles in various phospholipids and esters; apparent deficiencies in the activity of various peroxidases; imbalances in the metabolism of metals implicated as catalysts of the lipid peroxidation process; and data on the chemical structure of the lipopigment. These are reviewed below.

Fatty acid disturbances in ceroid-lipofuscinosis

(a) Lipids in blood. There have been a number of reports of changes in the fatty acid composition of complex lipids in the serum and plasma of people affected with the neuronal ceroid-lipofuscinoses. In a patient with infantile ceroid-lipofuscinosis, serum lecithin (phosphatidylcholine) had a raised content of arachidonic acid (20:4(n-6)). A sibling, also diagnosed as affected, showed no such elevation (Hagberg, et al., 1974). A similar pattern of arachidonate increase and linoleate decrease in serum phosphatidylcholine was reported in another patient with infantile ceroid-lipofuscinosis (Anzil, et al. 1976) but these workers questioned the relevance of these data to any primary underlying biochemical abnormality. Studies of total serum fatty acids in five patients with juvenile ceroid-lipofuscinosis showed a marked lowering of linoleate (18:2(n-6)) and a large increase in fatty acids with more than 20 carbons. However, "neurological controls", . "in-patients suffering from other nervous disorders" showed similar changes (Pronk, et al., 1973). A large reduction in leucocyte docosahexaenoic acid (22:6(n-3)) in patients with juvenile ceroid-lipofuscinosis has been reported and a defect in the metabolism of linolenic acid (18:3(n-3)) derived fatty

acids suggested (Pullarkat, et al., 1978). Nine patients with late infantile ceroid-lipofuscinosis had decreased serum linoleic acid and increased arachidonic, stearic (18:0), oleic (18:1(n-9)) and eicosatrienoic (20:3(n-9)) acid levels. These results were linked by the authors to the possible abnormal loss of polyunsaturated fatty acids through lipid peroxidation (Jensen, et al., 1978). Disturbed levels of serum lecithin, total leucocyte and total lymphocyte fatty acids have also been reported in some slightly unusual or poorly diagnosed forms of ceroid-lipofuscinosis (Becker, et al., 1979; Rumbach, et al., 1983; Vallat, et al., 1985). Plasma arachidonate and linolenate levels have been monitored in dogs affected with ceroid-lipofuscinosis, and compared with those of dogs heterozygous for the disease (Farnsworth, et al., 1982). Between 4 months and 18 - 20 months, the levels of phospholipid and cholesteryl linoleate were higher in the homozygous affected animals, while the arachidonate level was lower, the reverse of the general trend observed in the human studies above.

(b) Lipids in brain. There have been a number of reports of general lipid changes in the brains of people affected with ceroid-lipofuscinosis. The most notable general finding is an increase in esterified cholesterol in the infantile and late infantile forms of the disease (Van Dessel, et al., 1977; Bourre, et al., 1979; Hagberg, et al., 1968; Nevalainen, et al., 1973).

Specific changes in brain phospholipid fatty acids have also been reported. In the infantile form, fatty acid profiles of the major phospholipids, phosphatidylcholine, phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine have been recorded as differing from those of normal controls. In particular, there was an increase in 20:4(n-6), a decrease in 22:4(n-6) and a large decrease in 22:6(n-3). The name "polyunsaturated fatty acid lipidosis" was proposed to distinguish this disease from other forms of ceroid-lipofuscinosis in which these changes were not observed (Svennerholm, et al., 1975; Svennerholm, 1976). In contrast other workers reported a decrease in the proportion of 22:6(n-3) in grey matter phosphatidylserine in the infantile, late infantile, adult and 'pigment variant' forms (Jervis and Pullarkat, 1978; Pullarkat, et al., 1982), but no changes in the fatty acids of the other

phospholipids. The fatty acid composition of whole brains of dogs affected with ceroid-lipofuscinosis have also been analysed (Reddy, et al., 1985). No differences from controls were noted, but significantly lower microsomal acyl transferase activities were reported, particularly for the incorporation of arachidonic acid into phospholipids.

Peroxidase deficiencies in ceroid-lipofuscinosis.

The report of a deficiency in leucocyte peroxidase in a patient with late infantile ceroid-lipofuscinosis, and in two with the juvenile form of the disease (Armstrong, et al., 1973) was linked to the putative lipid peroxide nature of the stored lipopigment, and caused considerable excitement (Zeman, 1974). A number of communications quickly appeared, affirming a leucocyte peroxidase deficiency, or at least differences in the solubility of a peroxidase, in the late infantile (Armstrong, et al.,1974a; Awasthi, et al., 1977; Jensen, et al., 1977), juvenile (Armstrong, et al., 1974a; Clausen and Jensen, 1975; Pilz, et al., 1976a; Farrell and Sumi, 1977; Gadoth, 1978), and adult forms of the disease (Armstrong, et al., 1974 b; Bozdech, et al., 1980). The activity of a leucocyte peroxidase was also found to be low in English Setter dogs affected with ceroid-lipofuscinosis (Patel et al., 1974). Three of these reports of peroxidase abnormalities in the ceroid-lipofuscinoses questioned a primary role because a myelo-peroxidase deficiency had already been described in leucocytes, and was not associated with the clinical symptoms of ceroid-lipofuscinosis (Awasthi, et al., 1977; Pilz, et al., 1976a; Farrell and Sumi, 1977). Other reports found no evidence for a peroxidase deficiency in the infantile (Anzil, et al., 1975; Den Tandt and Martin, 1978; Becker et al., 1979) and juvenile (Haust et al., 1976; Den Tandt and Martin, 1978; Pilz, et al., 1978; Tsan et al., 1978) forms, and these workers rejected the idea of a peroxidase deficiency.

Other peroxidase activities have also been reported as abnormal in the ceroid-lipofuscinoses. Glutathione peroxidase activity has been measured as significantly lower in infantile (Westermarck and Sandholm, 1977; Jensen, et al., 1978) and late infantile (Jensen et al., 1978; Westermark and Sandholm, 1977) ceroid-lipofuscinosis and thyroid peroxidase activity reported as low in the late infantile and

juvenile forms (Armstrong et al., 1975). Peroxidase activity in the retinal pigment epithelium of affected English Setters has been monitored over the two year course of the disease. Retinal pigment epithelium peroxidase activity increases with age in normal dogs. It was higher than normal in affected young dogs but decreased with age (Armstrong et al., 1978). These differences in peroxidase activity were linked with differences in the subcellular distribution of the enzyme (Siakotos et al., 1978). Activity of plasma glutathione peroxidase in these animals followed a similar pattern of an apparently abnormal decline with age (Farnsworth et al., 1982). Workers who found glutathione peroxidase levels to be low in the infantile and late infantile forms of the disease found an elevated level of this enzyme in the juvenile form (Jensen and Clausen, 1982). People with juvenile ceroid-lipofuscinosis have normal lymphocyte and erythrocyte superoxide dismutase activities (Marklund and Plum, 1978).

Much of the debate as to the relevance of apparent peroxidase deficiencies to the pathogenesis of the ceroid-lipofuscinoses has centred on the methodology of measurement. Between 1974 and 1982 the requirements for the detection of low peroxidase activities narrowed to a strict set of otherwise arbitrary conditions and substrates (Armstrong, 1982). The issue became the distribution of the enzyme after subcellular fractionation, rather than total activity, but no balance sheet of total homogenate activity compared with the sum of activities in the various subcellular fractions has ever been presented. Hydrogen peroxide itself is a very good catalyst of the reaction measured, especially in a membrane suspension. No correction has ever been made for the kinetic consequences of assaying the transformation of lipophilic substrates in membrane suspensions (Parry et al., 1976). In short, these enzymes and their enzymology have not been well characterised. Attempts to characterise a particular protein with peroxidase activity that is deficient in tissues from patients affected with ceroid-lipofuscinosis have failed. Isoelectric focusing revealed no qualitative or quantitative differences in these enzymes in saliva (Pilz et al., 1976b), parotid gland (Pilz and Goebel, 1977), liver or brain (Garg et al., 1982).

Iron and copper imbalances in ceroid-lipofuscinosis

Recent reports state that the presence of iron or copper is necessary for lipid peroxidation to lead to fluorophors appropriate to lipofuscin formation (Gutteridge, 1984, 1985) and by implication to the formation of the lipopigment in the ceroid-lipofuscinoses. These contradict earlier reports of analogous fluorophor generation in the absence of copper or iron (Chio and Tappel, 1969 a, b).

Analysis of metals in lipopigment isolated from the brains of humans affected with ceroid-lipofuscinosis revealed copper, iron and calcium levels above those in age pigment, (Siakotas and Koppang, 1973). Other workers have failed to confirm this high iron content (Johansson, et al., 1984). Non-protein-bound iron has been reported as elevated in cerebrospinal fluid from patients affected with both the juvenile and infantile forms of ceroid-lipofuscinosis. This apparent increased iron content has been linked to a decreased ability of the cerebrospinal fluid to inhibit hydroxyl radical production, a necessary step in lipid peroxidation (Gutteridge, et al., 1982a). Total iron in the cerebrospinal fluid of patients with infantile and juvenile ceroid-lipofuscinosis has also been reported to be elevated (Johansson, et al., 1984).

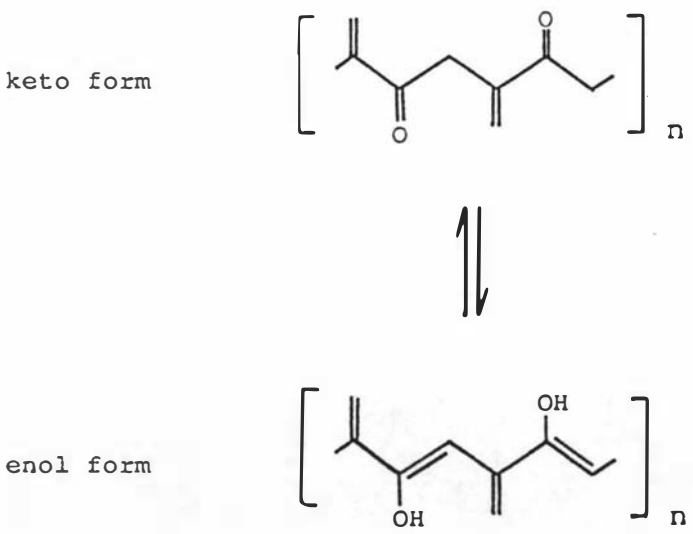
It has been proposed that the raised iron concentrations in cerebrospinal fluid of patients affected with ceroid-lipofuscinosis may arise from a defect in iron metabolism and lead to a consequent decrease in protection against its damaging oxidative effects (Gutteridge, et al., 1983).

The chemical nature of the lipopigment

Analyses of the lipopigment isolated from the brains of human patients with ceroid-lipofuscinosis have concentrated on the nature of the fluorophor proposed to be responsible for the fluorescent properties of the lipopigment. Early studies of the lipid components of the lipopigment disclosed a highly acidic structure that was fluorescent and did not migrate in normal phospholipid thin layer chromatography developing solvents (Siakotos, et al., 1972; Siakotos and Koppang, 1973; Zeman, 1976). Its structure was designated to be the highly fluorescent Schiff base polymer consequent to lipid peroxidation (see Fig. 1), because of its similarities in fluorescent and

chromatographic properties to material generated in in vitro peroxidation studies (Elleder, 1981). However no systematic chemical characterisation of the material derived from the lipopigment has been reported. A second fluorescent polymer was found in the neutral lipids of lipopigment (Siakotos, et al., 1972; Siakotos and Koppang, 1973; Zeman, 1976). It has subsequently been designated as "polymalonaldehyde" on the basis of the similarities of its chromatographic and fluoroscent properties compared to in vitro generated so called "polymalonaldehyde" (Gutteridge, et al., 1977; Siakotos and Munkres, 1981; Gutteridge, et al., 1982b). In this case the structural assignment is even more unclear as the in vitro generated material has not been adequately characterised. A tentative structure has been proposed (Gutteridge, et al., 1982b) (Fig. 2). This unsaturated polyketone is a highly polar structure, especially as extensive conjugation favours the enol tautomer. It is in effect a polyalcohol and could not have the chromatographic properties ascribed to it.

Fig. 2. Proposed structure of "polymalonaldehyde"



Other workers failed to confirm the presence of the "highly acidic polymer" in lipid extracts from brains of patients affected with ceroid-lipofuscinosis. Instead, a number of "lipoid fluorophors" were found, five in the neutral lipids and two in the polar lipids. Normal brain extracts contained the same fluorophors albeit in lower concentrations as judged by the extent of their fluorescence (Garg, et al., 1981).

An alternative polymeric structure has been proposed for "the fluorophor" in the ceroid-lipofuscinoses. Infra-red spectra and chemical studies of the fluorescent residue left after extraction of lipopigment isolated from a brain affected with late-infantile ceroid-lipofuscinosis, suggested an unsaturated polyamide structure (Wolfe, et al., 1976). Further analysis using infra-red, nuclear magnetic resonance and mass spectroscopy combined with partial base hydrolysis studies lead to the postulation of a major retinoyl component, possibly complexed to a small peptide (Wolfe, et al., 1977). Even these analyses and assignments were ambiguous. They were quickly questioned, as the data were also compatible with a cholesterol and/or a retinol-cholesterol complex (Nelson and Halley, 1977).

Two attempts at the direct demonstration of peroxidised lipids in the lipopigment have been reported. A particular modification and elongation of the Dam reaction for peroxides was positive in tissue paraffin sections of brains from dogs affected with ceroid-lipofuscinosis, but the normal Dam procedure was negative (Armstrong and Koppang, 1982). Recent electron spin resonance studies of lipopigment isolated from dogs affected with ceroid-lipofuscinosis failed to reveal any active oxygen species, and thus gave no indication of a primary role for lipid peroxidation in ceroid-lipofuscinosis (Vistnes, et al., 1983).

A critique of the peroxidation theory

Despite the persistent evocation of a role for abnormal peroxidation in ceroid-lipofuscinosis, no rigorous mechanism for this process has ever been proposed. In addition to the conflicting reports for each of the pieces of evidence alluded to above, there are problems in fitting each into a coherent mechanism. There is little dispute that the lipopigment is in some way related to lysosomes, but it is unclear

at which stage peroxidised material is supposed to enter lysosomes, or as to whether the postulated abnormal peroxidation takes place within these structures. Disturbances in fatty acid profiles of various lipids in either blood or brain could be a consequence of peroxidation either inside or outside lysosomes. If one accepts that leucocyte peroxidases are involved in protection against oxidative damage, deficiencies would argue for the peroxidation taking place within lysosomes. On the other hand, glutathione peroxidase is not a lysosomal enzyme and if a deficiency of this enzyme is involved it would lead to the abnormal extralysosomal occurrence of peroxidation products. If copper and iron are centrally involved in the peroxidation reactions required for the generation of the fluorophor, then this process would take place in the nascent lipopigment. On the other hand, the failure to find active oxygen radicals or obtain a strong peroxide staining reaction in these structures suggests that they would have to be the final depositories of the peroxidised material.

Each piece of supporting evidence for peroxidation is also open to other explanations. Differences in brain phospholipid fatty acid profiles might be a consequence of brain atrophy. In addition these molecules are not protected against degradation by freezing, and prolonged post-mortem storage could also result in the loss of polyunsaturated fatty acids.

Leucocyte peroxidase is not generally thought to be involved in protection against free radical damage initiated by active oxygen species, rather the opposite. Its function is to supply these species for antimicrobial activity via the respiratory burst. Some people are deficient in leucocyte peroxidase activity. They suffer some increased susceptibility to infection but do not develop ceroid-lipofuscinosis (Gabig, 1980).

The normal function of glutathione peroxidase is to reduce hydrogen peroxide in erythrocytes, avoiding the non-enzymatic formation of methemoglobin but it does not seem to be an important enzyme. Homozygous deficiency is common in Mediterranean peoples, and affects 30% of people of Jewish ancestry, who live with no apparent ill effects (Beutler,1979). One of the functions of

lysosomes is to regulate the homeostasis of metals, by sequestering excesses and in this capacity they are centrally involved in the metabolism of iron and copper (Sternlieb and Goldfischer, 1976). Overload of this system has been observed in animals and does not lead to slow degenerative changes, but to acute effects. Copper poisoned sheep suffer acute centrilobular hepatic necrosis and a haemolytic crisis (Gooneratne et al., 1979). Iron given therapeutically to piglets to prevent anaemia precipitates massive liver necrosis in those with inadequate Vitamin E (Kelly, 1985).

Many of the claimed properties of "the fluorophor" in the ceroid-lipofuscinoses are the same as in vitro oxidation artefacts which can occur during lipid isolation and characterisation. Good lipid chemistry requires that extractions be performed on fresh tissue and subsequent manipulations carried out rapidly and with the minimum exposure to air, light and heat. Unless sufficient care is taken, artefacts will form, many of them highly fluorescent. Work with human derived lipopigment is inevitably hampered by delay inherent with tissues obtained at post mortem of human cadavers. Under these circumstances, autolysis and in vitro oxidation of lipids is likely to result in the formation of artefacts.

Other postulates on the pathogenesis of the ceroid-lipofuscinoses

Lesions in biochemical pathways that do not involve lipid peroxidation, have also been alluded to as possibly being responsible for the ceroid-lipofuscinoses. The name "polyunsaturated fatty acid lipidosis" was coined for an infantile form, based on abnormal brain phospholipid fatty acid profiles. A disturbance in "the further metabolism of arachidonic acid" was proposed as the primary lesion (Svennerholm, 1976). Because of the reported apparent specificity of phospholipid fatty acid changes in phosphatidylserine in all forms of the disease, a possible defect in the metabolism of this compound has also been suggested (Pullarkat et al., 1982). No further delineation of either proposition has been forthcoming, and no explanation has arisen for the apparently contradictory nature of these results.

The reported retinoid nature of the fluorophor in the lipopigment gave rise to the hypothesis that "the gene defect in Batten's disease

involves an enzyme or enzymes that catabolise retinoic acid" (Wolfe, et al., 1977). More recently another polyisoprenoid, dolichol, has been reported as a major component of isolated cytosomes (Wolfe, et al., 1982 a; Ng Ying Kin, et al., 1983) and it suggested that the genetic defect may be associated with both retinol and dolichol metabolism (Wolfe, et al., 1982a). However these two compounds do not have a great deal in common, being trans and cis isoprenoids respectively. Dolichol levels in affected brains have been reported as elevated, as has the dolichol level in urinary sediments (Ng Ying Kin, et al., 1983; Ng Ying Kin and Wolfe, 1982; Wolfe, et al., 1983) and also in the cerebral cortex of people with Alzheimer's disease (Wolfe, et al., 1982 b). Chronic alcoholics also have elevated urinary dolichol levels (Pullarkat and Raguthu, 1985). Another group of workers found no increase in dolichol in urine from patients with ceroid-lipofuscinosis (Bennett, et al., 1985). Fibroblasts cultured from patients affected with ceroid-lipofuscinosis showed no differences from normal fibroblasts in their incorporation of acetate and mevalonolactone into dolichol, nor in the metabolism of this compound and thus "no evidence was found to support the hypothesis that Batten's disease is due to a defect in dolichol metabolism" (Paton and Poulos, 1984). Recent studies have suggested that dolichyl phosphates may be the important component elevated in brains of patients with ceroid-lipofuscinosis (Hall and Patrick, 1985) and in affected English Setters (Keller, et al., 1984). These workers suggested that the ceroid-lipofuscinosis might involve a defect in the metabolism of dolichol-linked oligosaccharides.

Concluding remarks

Despite the extensive literature reviewed above, little is actually known about the biochemistry of the ceroid-lipofuscinoses. None of the proposed defects is supported by definitive experimental evidence neither have any led to an acceptable diagnostic procedure. This still depends on clinicopathological examinations. Nevertheless various treatments to alleviate or cure ceroid-lipofuscinosis have been suggested and/or tried.

"Antioxidant therapy" dependant on dietary vitamin E supplementation, dietary butylated hydroxytoluene and methionine has been administered

to patients for several years, but after initially favourable reports, has been judged to "only retard the progress of the disease but not cure it" (Zeman, 1974; Santavuori and Westermarck, 1984). Dietary selenium has also been administered to overcome the "glutathione peroxidase deficiency". Recently desferrioxamine, a chelator of free iron has been added to the antioxidant therapy (Westermarck and Santavuori, 1984). It has been suggested that a diet low in vitamin A and carotene ".....may arrest, delay or prevent the progress of the disease" (Wolfe, et al., 1981), but there is no report of this being tried. It is doubtful if any of these treatments are any more effective than that of Stengel's patients who "were moved in order that goblins or whatever other creatures from the underworld" were not able to exert any power (Stengel, 1826).

The genetics of the disease, i.e. autosomal recessive, suggests pathogenesis is associated with an enzyme deficiency, rather than a structural protein abnormality. The various forms and subtypes of this group of diseases and the close homotypism and homochronism between affected siblings suggest a number of mutations leading to either different alterations in the same protein, or alterations of different proteins with related activities. In other inherited storage diseases, the enzyme deficiency has been deduced from the nature of the dominantly stored chemical species and the same is true for many other enzymeopathies. Understanding has arisen when the structure of the abnormally prevalent compound has been determined, and possible enzymes responsible for its metabolism or degradation assayed for.

Whatever the relevance of lipopigment bodies to the development of clinical signs in the ceroid-lipofuscinoses, they must themselves be related to the underlying enzyme defect, yet no systematic analysis of their composition has been reported. The dominantly stored chemical species in a storage disease is usually present in such amounts that it can also be detected in tissues without recourse to subcellular fractionation.

This thesis describes methods of isolation and the systematic analysis of lipopigment from various tissues of sheep affected with ceroid-lipofuscinosis. In addition, analyses of total tissues are included.

Much of this work has been carried out on visceral organs, as the function of these is not apparently affected by the disease and the problem of delineating primary and secondary changes does not arise as it does with brain which becomes severely atrophied as the disease progresses. Because of the historical connections of the ceroid-lipofuscinoses with the lipidoses, and the number of proposed defects in lipid metabolism outlined above, initial emphasis was placed on this group of compounds.

CHAPTER II

MATERIALS AND METHODS

Animals and tissues

Sheep with ceroid-lipofuscinosis were bred in an experimental flock of heterozygous ewes mated to 8 - 9 month old affected rams. Diagnosis was established by histopathology of brain biopsies at 2-4 months of age (Jolly, et al., 1980). Tissues were obtained from affected sheep 12 - 24 months old and chilled on ice within minutes of euthanasia. Control tissues were obtained from age matched normal Southdown or New Zealand Romney sheep husbanded under similar conditions except that affected sheep were housed from 20 months of age.

Cerebrospinal fluid (CSF) was obtained from the cisterna magna of sheep anaesthetised with sodium thiopentone and placed in lateral recumbency. About 4 ml was obtained from each animal. CSF samples were centrifuged at 2,000g max for 5 min within 20 min of isolation. Measured volumes of CSF were then freeze dried for metal analyses.

Lipid extractions and characterisations

(a) Total lipids: All lipid extractions were carried out as quickly as possible in the absence of heat and light under a photographic safelight (Kodak, Wratten Series filter OB) on fresh tissues or lipopigment isolated from fresh tissue. Lipids were extracted from liver, kidney and pancreas by a modified Bligh and Dyer procedure (Palmer, 1971). They were extracted from isolated lipopigments and grey matter dissected from the cerebral cortex, by the method of Folch et al. (1957). Unless otherwise stated the extraction solvents used in both procedures contained 0.05% w/v butylated hydroxytoluene (BHT). Lipids were either subject to further manipulation immediately, or they were stored in the dark dissolved in cyclohexane and sealed under nitrogen. Total lipids were quantitated by drying to constant weight in a desiccator over silica gel, after extraction with solvents containing no BHT.

(b) Phospholipids: Phospholipids were separated from neutral lipids by acetone precipitation of the former (Kates, 1972). Phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and phosphatidylcholine were separated by thin-layer chromatography (TLC) on 20 x 20 cm x 0.6 mm silica gel plates (type GF, Sigma Chemical Co.) in chloroform:methanol:acetic acid:water; 50:25:4:2* (Skipski, et al., 1964). All phospholipids were detected by either staining the plate in iodine vapour, spraying with a 1% solution of iodine, quenching of the 254 nm induced fluorescence of the silica gel, or staining with a modified Dittmer-Lester spray (Vaskovsky and Kostetsky, 1968). Phosphatidylcholine and sphingomyelin were selectively detected by their reaction to Dragendorff's reagent (Bregoff, et al., 1953) and phosphatidylserine and phosphatidyl-ethanolamine by their selective staining with a solution of 0.2% ninhydrin in n-butanol saturated with water, and subsequent warming of the TLC plate. Bis(monoacylglyceryl)phosphate [$(MAG)_2P$] was characterised by two dimensional TLC. The developing solvents were chloroform:methanol:acetic acid:water; 50:25:4:2 and then chloroform:methanol:ammonia:water; 65:20:2:2 (Wherret and Huterer, 1972). $(MAG)_2P$ was subsequently separated from the other phospholipids by TLC in chloroform:methanol:ammonia:water; 65:20:2:2 (Joutti et al., 1976). Phospholipids were quantitated by the method of Raheja et al. (1973), after TLC separation.

(c) Neutral lipids: Neutral lipids were separated by TLC on "Baker-flex" silica gel IB-F plates (J.T. Baker & Co.), developed in n-hexane:ether:acetic acid; 80:20:1, and stained with iodine vapour. Individual components were identified by co-chromatography of standards. Anisaldehyde stained for isoprenoids (McSweeney, 1965) and ferric chloride for cholesterol and cholesteryl esters (Lowry, 1968). Retinol and retinyl esters were identified by their fluorescence and quantitated colourimetrically using tricholoracetic acid in chloroform (Bayfield, 1975). This reagent was also used as a spray-on stain for TLC plates. Cholesterol, dolichol and ubiquinone were also characterised by normal phase high-performance liquid chromatography (HPLC) on a cyanopropyl column eluted with 0.1%

* All solvent ratios given in this thesis are volume to volume.

isopropanol in hexane (Palmer et al., 1984). The chain lengths of dolichol were established by reverse phase HPLC on a C18 column (Ng Ying Kin et al., 1983). Preparative TLC on 20 x 20 cm x 0.6 mm plates developed in n-hexane:ether:acetic acid; 80:20:1, or benzene, was used to isolate individual neutral lipids that were detected by staining a side band with 1% iodine in chloroform.

(d) Methanolysis of bands 1 and 2: The lipids designated as bands 1 and 2 (Fig.3, Chapter III) were separated from 20 mg of lipopigment neutral lipids by preparative TLC developed with benzene. The silica gel containing them was incubated with 2 ml of 2M sodium methoxide in 8 ml of benzene at 20°C for 45 min, neutralised with HCl, 5 ml of water added, and the top layer taken and analysed by TLC and HPLC as described.

(e) NMR: ^{1}H and ^{13}C NMR spectra were recorded as $CDCl_3$ solutions at 32°C in 5mm tubes on a Varian CFT 20 spectrometer, operating at 80 MHz for ^{1}H and 20 MHz for ^{13}C observations. Tetramethylsilane (TMS) was used as an internal standard.

Preparation and analysis of fatty acid methyl esters

(a) Transmethylation: Fatty acid compositions were obtained by gas-liquid chromatography of the fatty acid methyl esters formed by transmethylation of the parent compounds separated by TLC as described above. Side bands of the developed TLC plates were stained with iodine to identify the appropriate lipid and the silica gel containing that compound scraped from the rest of the plate. Fatty acid methyl esters were prepared from phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylcholine and triglycerides by incubation of the silica gel containing them in 7.5 ml of 0.5 M sodium methoxide at 20°C for 30 min, and extracted with 7.5 ml of n-hexane. Fatty acid methyl esters were prepared from $(MAG)_2P$ by reflux of the silica gel containing it in 7.5ml of 2% H_2SO_4 in methanol for 2h, and extracted with 7.5ml of n-hexane. Free fatty acids and dolichol were isolated together by preparative TLC. The fatty acids were methylated with freshly distilled ethereal boron trifluoride (Morrison and Smith, 1964) and the resultant fatty acid methyl esters isolated by preparative TLC.

(b) Gas-liquid chromatography: The extracted methyl esters were analysed by gas liquid chromatography on a Silar 7-CP support coated open tubular glass column, 50m x 0.5mm internal diameter, (Scientific Glass Engineering Pty. Ltd. [S.G.E], North Melbourne, Australia) in a Varian 1440 Aerograph (Varian, Walnut Creek, Calif.) converted to capillary column use with an S.G.E. Unijector. The column temperature was 170°C, the injector temperature 200°C and the detector temperature 250°C. The gas (N_2) flow rate through the column was 2.5 ml/min. The fatty acid esters were detected with a Varian flame ionisation detector and quantitated by the product of peak height and retention time (Carroll, 1961).

(c) Equivalent chain lengths: Equivalent chain lengths (ECLs) were calculated from the relative retention times of 16:0 and 18:0 methyl esters by the following equation

$$ECL = \frac{a\ (\text{Log}\ R_x\ -\ \text{Log}R_n)}{\text{Log}\ R_{n+a}\ -\ \text{Log}\ R_n} + n$$

where R = retention time
n = the number of carbons in one reference fatty acid
n + a = the number of carbons in the other reference fatty acid
x = the fatty acid of unknown structure

Tentative assignments were made by the comparison of ECLs with tabulated values (Jamieson, 1975; Ackman and Eaton, 1978).

(d) Argentation chromatography: Selected samples of fatty acid methyl ester mixtures were separated into classes dependent on the number and nature of their double bonds by argentation TLC (Morris, 1966). Fatty acid methyl esters were separated on 25 x 10 cm x 0.6 mm silica gel TLC plates impregnated with 14% w/w silver nitrate, developed in n-hexane:ether; 9:1, and visualised by spraying the plates with 0.2% 2'7' dichlorofluorescein in ethanol, and exposure to

254 nm ultraviolet light. Fatty acid methyl esters showed as yellow bands against a dark red background. The silica gel containing the fatty acid methyl esters was scraped from the plate, suspended in 8 ml of 1% sodium chloride in methanol:water; 7:3, and extracted twice with 4 ml of n-hexane. The regions on the TLC plate between the obvious bands were also extracted in the same way.

(e) Hydrogenation: Fatty acid methyl esters prepared from an isolated phospholipid or from particular argentation bands were dissolved in 2 ml of methanol in a glass centrifuge tube and 100 mg of activated palladium on charcoal added. The tube was placed in a Parr bomb hydrogenator, purged five times with hydrogen and then shaken under 40 pounds per square inch of hydrogen for 3 h. Methanol was then added to make a final volume of 5 ml and the fatty acid methyl esters extracted twice with 3 ml of n-hexane.

Isolation and characterisation of lipopigment bodies from liver using pronase

(a) Isolation: The isolation of lipopigment bodies commenced within 1 h of euthanasia. All procedures were carried out in darkness or under a photographic safelight (Kodak, Wratten Series filter OB). Liver, 100g, was homogenised in 200 ml of ice-cold buffer (0.8M sucrose, 10mM KCl, 50mM Tris, 10mM 2-mercaptoethanol, 1mM EDTA, pH 7.5) for 2 min in a Sorvall Omnimixer (Ivan Sorvall Inc.), filtered through gauze, made up to 500 ml with buffer and centrifuged at 27,500g max x 30 min at 4°C, in a GSA rotor on a Sorval RC-5 centrifuge. The pellet was resuspended in 20 ml of 2M sucrose and centrifuged as above. The resultant pellet was then resuspended in 150 ml of water and 150 mg of pronase added. The pH was adjusted to 7.5 with Tris base and the mixture incubated with stirring at room temperature for 60 min, after which it was filtered through glass wool. The specific gravity of the filtrate was adjusted to 1.18 by the addition of CsCl, and 2-mercaptoethanol added to 10mM. The lipopigment bodies were pelleted by centrifugation at 48,000g max x 90 min at 4°C in an SS-34 rotor.

(b) Microscopy: Samples were suspended in glycerol for immediate fluorescence microscopy, or fixed in 2% glutaraldehyde and 3% paraformaldehyde in 0.1M phosphate buffer, pH 7.2, and further

prepared for transmission electronmicroscopy by standard techniques (Jolly et al., 1980). The remainder was resuspended in 145 mM NaCl for immediate lipid extraction.

(c) Densities: The isopycnic density of the lipopigment bodies was determined by centrifugation on CsCl gradients (0.64-3.38M) and sucrose gradients (0.8-1.8M) at 70,000g max x 14h at 4°C, in a Beckman SW 25.1 rotor.

(d) Fluorescence of lipopigment lipids: Lipid extractions of lipopigment bodies, and extractions of control and affected total liver lipids were carried out as quickly as possible in the absence of BHT. The fluorescent spectra of the lower chloroform phases of these extractions were determined on an SPF-500 ratio spectrofluorometer (American Instruments Co.). Aliquots were diluted with chloroform:methanol; 2:1 to give similar maximum emission intensities, that were linearly related to concentration. The weight of lipid necessary for this intensity was determined by drying it to constant weight.

Aliquots of the chloroform phases of the lipid extractions were also loaded onto a 20 x 20cm x 0.6mm silica gel TLC plate at 2mg of lipid/cm. The plate was developed to 5cm in chloroform:methanol; 1:1, allowed to dry, developed to 10cm in ether:acetic acid; 99:1, allowed to dry, developed to 20cm in n-hexane:ether:acetic acid; 80:20:1, and allowed to dry. It was then irradiated at 350 nm and the fluorescence photographed through a yellow filter on Ektachrome 64 ASA film. The plate was then stained with iodine vapour. All operations were carried out as quickly as possible in darkness or under the photographic safelight.

(e) Determination of the non-lipid content of lipopigment bodies: The dry weights of the lipopigment bodies, the weights of the lipids and the weights of the insoluble residues after lipid extractions of lipopigment bodies, carried out in the absence of BHT, were determined by drying to constant weight in a desiccator over silica gel. Protein determinations were carried out on aliquots of the lipopigment bodies by the method of Lowry et al (1951) using bovine serum albumin as a standard.

Isolation and characterisation of lipopigment from liver, kidney, pancreas and brain without using pronase

(a) Isolation from liver or kidney: Liver or kidney cortex , 40g, was rinsed with 0.145 M NaCl and homogenised in 400 ml of ice-cold buffer (0.25M sucrose, 25mM KCl, 50 mM TrisHCl, 5mM $MgCl_2$, 10 mM 2-mercaptoethanol, pH 7.4) for 1 min in a Sorvall Omnimixer (Ivan Sorvall Inc.). The homogenate was filtered through gauze, centrifuged in a Sorvall GLC-1 centrifuge for 10 min at 70g max, and the supernatant centrifuged for 20 min at 1,400g max. The resultant pellet was resuspended in 100 ml of 0.4 mM Tris HCl, pH 7.4, sonicated for 1 min and centrifuged at 1,400g max for 20 min. The pellet was then resuspended in 120 ml of water and filtered through glass wool. Lipopigment bodies were harvested from the filtrate by centrifugation at 5,900g max for 20 min. All operations were carried out at 4°C.

(b) Isolation from pancreas: Lipopigment bodies were prepared from pancreas, 20g, in the same way as from liver and kidney, except that all centrifugations were for 30 min and the final harvesting centrifugation was at 2,800g max for 30 min.

(c) Isolation from brain: Grey matter, 25g, was homogenised in 250 ml of 0.4 mM Tris HCl, pH 7.4 for 2 min in a Sorvall omnimixer, filtered through gauze, sonicated for 2 min and centrifuged at 5,900g max for 10 min in an SS34 rotor on a Sorvall RC-5 centrifuge. The pellet was resuspended in 200 ml of the above buffer, sonicated again and the specific gravity of the suspension raised to 1.15 by the addition of CsCl to 17.5% w/w. This suspension was then centrifuged at 12,100g max for 30 min. The pellet was resuspended in 50 ml of 0.145M NaCl, and filtered through glass wool. The lipopigment bodies were harvested by centrifugation in a Sorvall GC-1 centrifuge at 1,400g max for 30 min. All operations were carried out at 4°C.

(d) Characterisation: Fluorescent microscopy and electron microscopy was carried out as described for liver lipopigment isolated with the use of pronase. Isolated lipopigment bodies were suspended in water and protein determined using bovine serum albumin as a standard (Lowry et al., 1951). Aliquots were immediately taken for the

analyses of the lipopigment components or stored frozen until used.

(e) Inhibition of lysosomal proteases: In order to inhibit the potential activities of lysosomal serine and thiol proteases, 5 mM phenylmethanesulphonylfluoride and 2.5 mM iodoacetate were added to the 0.4 mM Tris buffer used in the isolation of liver lipopigment bodies. After sonication the suspension was left to stand at 4°C for 20 min, prior to glass wool filtration. Liver lipopigment bodies were also prepared in the presence, and the absence, of 10 mM EDTA and 10 mM 2-mercaptoethanol to determine the possible effects of metal dependent and thiol activated proteases.

(f) Quantitative analyses of lipopigment components: The proportions of protein and lipid in the lipopigment bodies were determined as described above, except that the total lipopigment mass was determined by the sum of the lipid mass, the insoluble residue, and the non-volatile residue of the upper phase of the lipid extraction. Cholesterol, dolichol and ubiquinone levels were determined by quantitative normal phase HPLC on a cyanopropyl column eluted with 0.1% isopropanol in hexane, a method developed during the course of this work for this purpose (Palmer et al., 1984).

(g) Quantitative amino acid analyses: Lipopigment bodies were delipidated with n-butanol (Mecham and Mohammad, 1955) and hydrolysed in vacuo in 6 M HCl at 110°C for 16h. The resultant amino acids were analysed on a Beckman 119 BL amino acid analyser.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

(a) SDS-PAGE: SDS-15% 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 bisacrylamide 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. Freshly prepared 50 µl samples containing the appropriate amount of protein were loaded to each lane of the gel.

Fresh or once frozen lipopigment bodies and total homogenates were dissolved in a fresh solution of 1% w/v SDS and 5% v/v 2-mercaptoethanol at a concentration of 2mg of protein/ml. Aliquots were diluted with water and a boiled solution of glycerol and bromophenol blue to a final concentration of 12% glycerol and 6 µg/ml of bromophenol blue; and so that 50 µl contained 10 µg of lipopigment protein or 30 µg of total honmogenate protein. Samples were not heated at any stage. Electrophoresis was carried out at constant current, 8mA, at 4°C, until the bromophenol blue reached the bottom of the gel, in approximately 17 h.

Molecular weights of proteins were determined by the comparison of their migration rates with those of molecular weight standards.

(b) Silver stains: Gels were fixed for 1h in 12% w/v trichloroacetic acid and then silver stained with Bio-Rad silver stain kit (Cat. No. 161-0443), according to the instructions. They were then washed with 1L of 3.5% v/v acetic acid for 1 h and then 300 ml of a freshly prepared saturated solution of sodium diethyldithiocarbamate for a further 1h. The latter was removed by washing five times with 1L of water for 20 min. The gels were then further washed with 200 ml of 0.25% w/v silver nitrate solution for 30 min, rinsed with 400 ml of water and developed with three sequential aliquots of the Bio-Rad silver stain kit developer. The first development was for 1 min, the second for 5 min and the third until the desired staining intensity was reached. The staining reaction was stopped by washing the gel with 400 ml of 5% v/v acetic acid for 5 min. Gels were then kept in water or moist in air-tight bags. All steps after the addition of the silver nitrate solution were carried out under a photographic safelight (Kodak, Wratten Series filter OB). This staining was also obtained with a single silver nitrate wash by adding a diethyldithiocarbamate wash as above after the oxidiser wash in the Bio-Rad silver stain kit method, and thoroughly removing it with water before proceeding with the rest of the Bio-Rad silver stain method.

(c) Other stains: Gels were also stained for protein with 0.05% Coomassie Brilliant Blue R in methanol:acetic acid:water; 10:7:83 v/v for 30 min, and with amido black (Wilson, 1979). Glycoproteins were stained for by the periodic Schiff reaction (Gordon, 1975) and

Toluidine Blue was used as an RNA stain (Drysdale and Righetti, 1972).

Protease digestion of lipopigment bodies

Lipopigment bodies from all the tissues were incubated at 5mg of protein/ml in 100 mM Tris, 100 mM EDTA, 1% SDS, pH 7.5 and 1 mg/ml nuclease free protease at 37°C for 105 min. A further 1 mg/ml aliquot of protease was then added and the incubation continued for a further 90 min. Samples of the incubates were then subjected to SDS-PAGE.

Metal analyses

Freeze dried aliquots of the lipopigment that had been washed 2x with deionised water were weighed to ±0.02mg. Each sample weighed 5-40mg. They were then ashed in borosilicate test tubes at 500°C for 16h. The ash was dissolved in a measured volume, 4-8ml, of a 2M solution of redistilled analytical grade concentrated hydrochloric acid and multi-element analysis carried out on an Applied Research Laboratories plasma emission spectrometer. Copper levels in selected solutions were checked on an Instrumentation Laboratory IL 457 atomic absorption spectrophotometer. Total control and affected tissues, 1-2g wet weight, were also freeze dried, weighed and their metal contents analysed. The metal contents of known volumes of freeze dried CSF were analysed in the same way.

Chemicals

Type GF silica gel for TLC, type IV retinyl palmitate, ubiquinone 50, grade II porcine dolichol, chromatography standard grade cholesterol, linolenic acid, 2-mercaptoethanol, Coomassie Brilliant Blue R-250, phenylmethanesulphonylfluoride and sodium acetate were obtained from Sigma (St. Louis, Mo.). Molecular weight standards were obtained from Sigma (Kit No. MW-SDS 70 L), and also by the cleavage of Sigma Type 1 equine skeletal muscle myoglobin with cyanogen bromide, by a previously described method (Edmundson, 1963). Pronase Cat. No. 53702, activity 45,000 PUK/g, and nuclease-free protease (Pronase Cat. No. 537088) were obtained from Calbiochem-Behring (La Jolla, Ca.). Synthetic crystalline vitamin A alcohol was obtained from Koch-Light

Laboratories (Colnbrook, Slough, Berks., England). BHT and 45% boron trifluoride diethyletherate were obtained from BDH (Poole, England). All chemicals required for PAGE and subsequent silver staining were obtained from Bio-Rad (Richmond, Ca.), except for SDS, sucrose, sodium diethyldithiocarbamate and trichloroacetic acid that were also obtained from BDH and silver nitrate that was obtained from the Peking Chemical Co. (China). All water was filtered through a Milli-Q Reagent water system and a Millistak GS filter (Millipore Corp. Bedford, Ma.) so that it had a minimum resistance of 10 M ohms/cm. All other reagents were analytical grade and all solvents were double distilled.

CHAPTER III

NEUTRAL LIPIDS, PHOSPHOLIPIDS AND FATTY ACIDS IN LIVER AND BRAIN

Introduction

The morphological appearance and staining characteristics of the lipopigment indicate a lipoid nature. This has led to the development of the various hypotheses on pathogenic mechanisms outlined in Chapter I, involving different aspects of lipid metabolism. Each is supported by reported differences in the levels of certain lipids between control and affected brains. As liver is a major organ in lipid metabolism it should be a sensitive indicator of any lipid related metabolic defect. Accordingly total affected and control liver lipids were analysed as well as those of control and affected brains.

Because of the historical emphasis placed on polyunsaturated fatty acids, and because polyunsaturated fatty acids would be the species lost to lipid peroxidation, particular emphasis was placed on these molecules. Ruminants have a very restricted polyunsaturated fatty acid supply and any loss of polyunsaturated fatty acids would be more critical in sheep than in humans and could be expected to have more drastic consequences.

Results

(a) Liver total lipid contents: No significant difference was found in the yield of lipid extracted from control or affected liver, nor in the proportions of neutral and phospholipids (Table 2).

Total liver lipids were also extracted with BHT included in the extraction solvent, divided into neutral and phospholipids by acetone precipitation of the latter, and the individual lipid classes analysed.

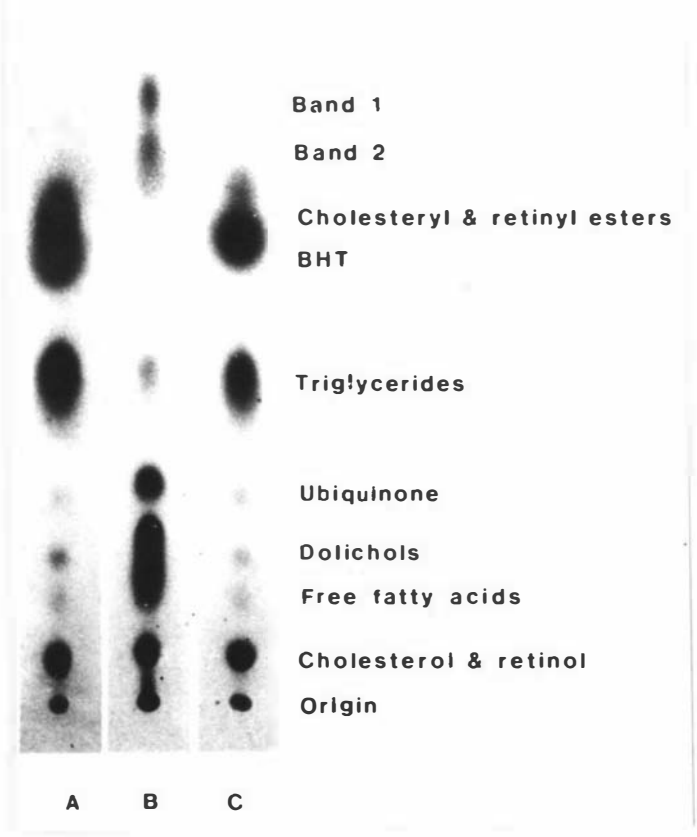
Table 2

Total Liver Lipids

The weight of lipid extracted from liver in mg/g wet weight of tissue ± the standard error of the mean with the number of experiments in parenthesis.

	Lipid extracted	% Phospholipid
Control liver	28.5 ± 2.73 (8)	72.6 ± 2.56 (6)
Affected liver	27.9 ± 3.63 (10)	73.6 ± 2.23 (6)

Fig. 3. TLC of neutral lipids. TLC of (A) control liver (B) isolated lipopigment and (C) affected liver neutral lipids developed in n-hexane:ether:acetic acic; 80:20:1, and stained with iodine. The isolated lipopigment neutral lipids did not contain BHT.



(b) Neutral lipids: No qualitative or gross quanititative differences were found in the distribution of species within the neutral lipids when these were separated by thin layer chromatography on silica gel (Fig. 3).

Retinol and retinyl esters were present in both control and affected sheep livers, as indicated by their staining properties and fluorescence. There was a wide animal to animal variation in the assayed levels of these compounds, all within the wide range of values previously reported for sheep liver (Bayfield, 1975) but no significant difference was found between the mean control and affected levels.

(c) Phospholipids: Both control and affected livers contained the same major phospholipid species, in quantitatively similar amounts (Table 3).

Table 3

Liver Phospholipid Composition

The distribution of phospholipid phosphorus between the major classes of phospholipid extracted from normal and affected liver and separated by TLC expressed as the mean percent of total phospholipid phosphorus ± standard error of the mean. The number of experiments is shown in parenthesis.

	Normal liver	Affected liver
Phosphatidylcholine	53.8 ± 4.11 (4)	55.3 ± 2.81 (3)
Phosphatidylinositol+ phosphatidylserine	11.4 ± 1.98 (3)	11.0 ± 1.50 (3)
Phosphatidylethanolamine	23.8 ± 3.49 (4)	26.8 ± 3.92 (3)
Remainder	10.6 ± 0.54 (3)	6.9 ± 5.80 (3)

(d) Fatty acids: The gas-liquid chromatography technique described gave base-line separation of most of the fatty acid methyl esters (Fig. 4).

(i) Fatty acids of liver: No significant differences were found between the fatty acid profiles of the major control and affected liver phospholipids, phosphatidylcholine and phosphatidylethanolamine (Table 4).

Trans 18:1(n-9), a component of both phospholipids, migrated faster than the monounsaturated fatty acids in argentation chromatography, and eluted as a faster running shoulder on cis 18:1(n-9) in the gas liquid chromatograms. No other trans fatty acids were detected. Traces of iso and anteiso 17:0 remained after hydrogenation of the fatty acid methyl esters, and eluted as part of 17:1s. The structure of 18:1(n-5) was assigned solely on the basis of its equivalent chain length. All other assignments were consistent with both argentation and hydrogenation data, which did not reveal any significant contamination with other fatty acid methyl esters.

There was a wide animal to animal variation in the fatty acid profiles of both control and affected liver triglycerides, but no significant differences (Student's t test) between the mean values from four control and four affected animals. The overall mean values for the major fatty acids were 16:0, 17.6%; 17:0, 2.3%; 18:0, 17.4%; 18:1(n-9), 30.3%; 18.2(n-6), 6.4%; 18:3(n-3), 4.2%; 20:1(n-9), 4.2%; 22:5(n-3), 3.0%. The large number of minor components detected included all the fatty acids present in the total tissue and lipopigment phospholipids, and additionally 18:2(n-9), 20:2(n-9), 22:2(n-9) and 22:3(n-6).

(ii) Of brain : The fatty acid composition of the major phospholipid classes of grey matter phosphatidylcholine, phosphatidylinositol phosphatidylserine and phosphatidylethanolamine from normal sheep were determined (Table 5). In addition to those fatty acids listed, 14:0, (0.8%), 15:0, (0.2%), 17:1, (0.2%), 18:3(n-9), (tr) and 18:4(n-3), (0.4%) were found in phosphatidylcholine. Hydrogenation of the phospholipid fatty acid methyl esters revealed

Fig.4. Gas-liquid chromatography of fatty acid methyl esters.

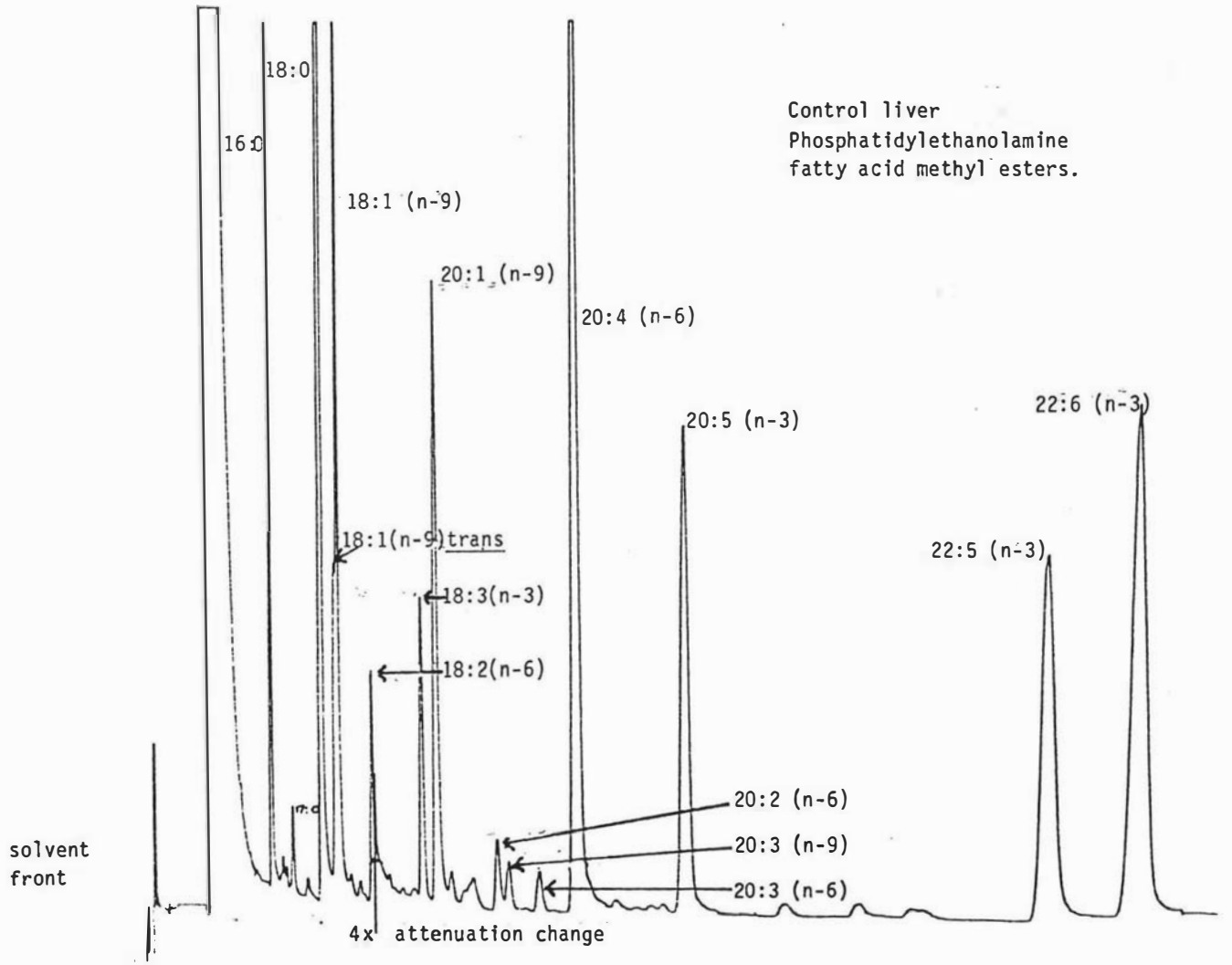


Table 4
Total liver phospholipid fatty acids

Mean percentage of fatty acids by weight from phosphatidylcholine and phosphatidylethanolamine from four normal and four affected livers. To facilitate presentation, standard errors are omitted but within each column means and standard errors were positively related. Estimated chain lengths (ECL) are the mean value of 21 determinations. Standard errors for these ranged from 0.011 for 17:0 to 0.081 for 22:6(n-3) fatty acids.

			Phosphatidylcholine		Phosphatidylethanolamine	
	Fatty acid	ECL	Control	Affected	Control	Affected
	16:0	defined	14.2	11.6	7.1	5.7
	16:1s	16.53	.6	.5	.7	.7
	17:0	17.01	.8	1.0	1.0	1.1
	17:1s	17.56	.2	.2	.3	.2
	18:0	defined	25.9	26.3	27.7	32.2
trans	18:1(n-9)	18.42	2.2	4.0	3.0	2.9
cis	18:1(n-9)	18.52	17.5	16.5	14.0	14.8
	18:1(n-5)	18.80	tr	tr	.5	.7
	19:0	19.01	tr	tr	.7	.5
	18:2(n-6)	19.29	5.3	6.2	3.9	4.5
	18:3(n-3)	20.21	2.2	3.6	2.4	2.5
	20:1(n-9)	20.44	3.0	2.4	2.3	2.0
	20:2(n-6)	21.26	.4	.6	.5	.5
	20:3(n-9)	21.40	.6	.6	.6	.5
	20:3(n-6)	21.72	.4	.5	.5	.3
	20:4(n-6)	22.13	4.2	4.5	10.8	9.2
	20:5(n-3)	23.03	5.2	6.0	7.5	6.2
	22:5(n-3)	24.96	6.9	6.6	6.7	6.1
	22:6(n-3)	25.38	8.8	7.8	9.4	8.7
	Total polyunsaturated		34.0	36.4	42.3	38.5

Table 5
Fatty acids in brain grey matter phospholipids

Data are the mean % by weight of fatty acids analysed for 4 sheep, except for the affected phosphatidylinositol values that arise from 3 sheep. To facilitate presentation, means are rounded to the nearest decimal place and standard errors are omitted. Within each column means and standard errors were positively related. Results were analysed by Student's t test. ECLs are the mean value of 37 determinations. Standard errors for these ranged from 0.042 for 22:6(n-3) to 0.010 for 18:1(n-9).

	Phosphatidylcholine		Phosphatidylinositol		Phosphatidylserine		Phosphatidylethanolamine		
Fatty Acid	Control	Affected	Control	Affected	Control	Affected	Control	Affected	ECL
16:0	48.4[a]	42.6[a]	6.8	10.0	3.1	2.4	4.2	5.7	defined
Complex 16:1s	.8	1.3	.2	.3	.4	tr	tr	tr	16.58
17:0	.3	.5	.3	.8	.3	.3	tr	.5	17.03
18:0	12.0	13.2	33.2	23.3	35.5	40.4	24.8	23.3	defined
18:1(n-9)+ 18:1(n-7)[c]	27.6	27.3	15.2	16.5	12.6	11.3	9.8[b]	13.7[b]	18.48
18:2(n-6)	.4[b]	.7[b]	.2	1.4	tr	.2	.2	.3	19.26
20:0	.1	.1	.4	.3	.3	.4	tr	tr	20.03
20:1(n-9) + 20:1(n-7)[c]	.9	1.2	1.1	1.5	1.3	1.2	1.0[a]	1.4[a]	20.41
20:2(n-9)	-	-	.4	.2	.2	.2	tr	.3	20.96
20:3(n-9)	.4	.6	1.3	1.6	1.5	1.5	2.0	2.5	21.30
20:3(n-6)	.3[b]	.5[b]	.5[a]	.7[a]	.6	.6	.5	.6	21.61
20:4(n-6)	2.6	3.4	8.5	12.2	9.1	9.2	9.9	10.8	21.98
20:4(n-3)	.1	.1	.5	.4	.4	.5	.3	.5	22.42
20:5(n-3)	.3	.3	.6	.6	.5	.4	.6	.6	22.88
22:3(n-9)	.1	.1	.6	.8	.8	.9	.8	.9	23.29
22:4(n-6)	.4[b]	.7[b]	2.6	4.8	3.3	2.9	4.9[a]	6.7[a]	23.93
22:5(n-6)	tr	tr	.6	.7	.7	.7	.5	.7	24.27
22:5(n-3)	.4	.6	2.3	3.0	2.6	1.8	3.9	4.1	24.79
22:6(n-3)	3.5	4.4	24.9	20.0	27.2	25.5	36.1[b]	27.3[b]	25.14

[a] P 0.01
[b] P 0.05
[c] 18:1(n-7) ran as a slower running shoulder on the 18:1(n-9) peak. The 20:1 peak was similarly split.

only traces of iso and anteiso 17:0 that were otherwise not fully resolved from 16:1, thus causing a complex peak on the chromatogram. No other branched chain acids, that could conceivably replace polyunsaturated fatty acids in phospholipid structures, were detected.

The differences in fatty acid profiles of phospholipids from sheep with ceroid-lipofuscinosis and normal controls were limited mainly to phosphatidylethanolamine. In affected sheep it contained elevated levels of 18:1(n-9), 20:1(n-9) and 22:4(n-6) but a lower 22:6(n-3) content. Some phosphatidylcholine n-6 fatty acids were also slightly enhanced in affected sheep at the expense of the 16:0 acid. No significant (Student's t test) differences were noted in the fatty acid profiles of control and affected grey matter phosphatidylinositol and phosphatidylserine.

Discussion

If the disease ovine ceroid-lipofuscinosis was a generalised lipidosis, then some increase in the amount of lipid in affected liver would be expected. This was not observed. No difference was observed in the proportions of neutral and phospholipids either, these being in agreement with values previously reported for New Zealand sheep liver (Peters and Smith, 1964). The similarity of dolichol levels in both control and affected liver militates against a generalised defect in its metabolism. The same is true of retinol. The wide non-disease related variation in the retinol contents observed occur because liver is a storage tissue for this compound, and levels are very sensitive to dietary intake (Bayfield, 1975).

There is no indication of any disturbance in the metabolism of any of the major liver phospholipids, nor in the fatty acid composition of phosphatidylcholine and phosphatidylethanolamine. Phosphatidylethanolamine is the phospholipid with the highest level of polyunsaturated fatty acids in mammalian membranes, yet even this compound shows no sign of any loss of polyunsaturated fatty acids which might be expected if there was a constant loss of these molecules caused by lipid peroxidation, particularly in a ruminant.

Differences in fatty acid profiles of control and affected grey matter

phospholipids are restricted to phosphatidylcholine and phosphatidylethanolamine, and are minor (Table 5). Differences of similar magnitude have been observed between foetal and developed human brain (Svennerholm, 1968; Svennerholm and Vanier, 1973). Changes in the fatty acid profile of phosphotidylethanolamine during normal brain development inversely parallel the differences between control and affected sheep brain phosphatidylethanolamine fatty acid profiles observed here. Although much less severe than the changes reported in brains affected by the infantile disease (Svennerholm et al., 1975; Svennerholm, 1976) the ovine differences are in the same direction. It should be noted in making the comparison that the human subjects had died of the disease whereas the sheep, although blind and with considerable brain atrophy, were euthanased at earlier stages. The quantitative differences in phosphatidylethanolamine fatty acids between normal and affected sheep are similar to those in brain biopsies from human patients at less advanced stages of the infantile disease (Svennerholm et al., 1975; Svennerholm, 1976).

When considered together with the normality of the fatty acid profiles of affected liver phosphatidylcholine and phosphatidylethanolamine (Table 4), the data presented above allow for no generalised disturbance in polyunsaturated fatty acid metabolism. The small differences observed in the sheep brain phosphatidylethanolamine fatty acids can be rationalised as consequential to brain atrophy, rather than being in any way connected to the primary disease process. Any large change in brain phospholipid fatty acid profiles in the ceroid-lipofuscinoses must therefore be a late event.

Pullarkat and colleagues have proposed an abnormality of phosphatidylserine metabolism for four forms of the disease based on studies of affected human brain phospholipid fatty acids (Pullarkat et al., 1982). In the present study the fatty acid profile of this phospholipid was similar in both normal and affected sheep and there was no significant reduction in its 22:6(n-3) content. Any change in phosphatidylserine fatty acids general to this group of diseases must therefore also be a late and secondary event.

The relatively small differences observed in brain phospholipid fatty acids between normal and affected sheep and the lack of any

disturbances in the liver phosphatidylcholine, phosphatidyl-ethanolamine and triglyceride levels of polyunsaturated fatty acids, argue against polyunsaturated fatty acids being lost to lipopigment formation. At the stages of the disease examined, affected brains contain large amounts of lipopigment, as do other tissues throughout the body. Lipopigment biogenesis is generalised and if there was a continual drain on the limited supply of certain unsaturated fatty acids which are highly conserved in the sheep, then one might expect greater changes in fatty acid profiles than are observed. Symptoms related to essential fatty acid deficiency do not occur in the diseased sheep. This calls into question the primary role of peroxidation of polyunsaturated fatty acids in lipopigment formation, at least in this form of ceroid-lipofuscinosis.

These total tissue studies do not support an involvement of any particular defect in lipid metabolism in ceroid-lipofuscinosis, but they do not definitively deny the possibility either. Accordingly it was decided to isolate purified lipopigment and systematically analyse its composition and properties.

Additional comments on the fatty acid composition of ruminant phospholipids

The data presented on the fatty acid composition of phospholipids from normal sheep are also of interest in their own right as they have not previously been reported. Sheep and other ruminants have a restricted polyunsaturated fatty acid supply. The levels of essential fatty acids available for brain development in the ruminant foetus and neonate is well below that available to other mammals (Noble and Shand, 1982) and bio-hydrogenation activity of rumen micro-organisms causes their essential fatty acid intake to remain severely restricted throughout life (Hawke and Silcock, 1969). It is such that would cause essential fatty acid deficiency in humans (Holman, 1978). How ruminants have adapted to this low essential fatty acid supply is not fully understood. Apart from trans 18:1(n-9), that is a product of rumen hydrogenation and would be incorporated into phospholipids as a consequence, there are no fatty acids in either phosphatidylcholine or phosphatidylethanolamine peculiar to ruminants, nor elevated levels of any species that might function as surrogate essential fatty acid derived components. Levels of the longer chain

polyunsaturated fatty acids in phosphatidylethanolamine are higher than in most ruminants and are similar to those reported for non-ruminant species (Crawford et al., 1976).

Most information on brain phospholipid fatty acids arises from studies of human brain, and the sheep show some differences most notably in phosphatidylinositol. A number of control processes depend on the distinctive metabolism of this molecule and the release of 20:4(n-6) from it (Nishizuka, 1983; Berridge, 1984). Sheep grey matter phosphatidylinositol contains only 8.5% of this fatty acid in contrast to 28.5% in human grey matter phosphatidylinositol (Svennerholm, 1968). The level of 18:3(n-3) derived fatty acids is high in sheep phosphatidylinositol where 22:6(n-3) accounts for 24.9% of all fatty acids compared to 4.6% in humans. These fatty acids are also elevated in the other sheep phospholipids relative to human values but to a lesser extent.

The sums of the 18:1(n-9) derived fatty acids are similar in both species for all phospholipids except phosphatidylinositol where the sheep content is higher. In sheep the level of 20:3(n-9), in all phospholipids, is at least twice that of humans, and might be indicative of fatty acid deficiency in non-ruminants (Holman, 1978). The related fatty acid 22:3(n-9) has been reported in humans (Sun, 1973) but not in sheep. However, despite the relatively elevated levels of these fatty acids in sheep they are not major fatty acids in any class of phospholipids. The 18:2(n-6) derived fatty acid content of phosphatidylserine is higher in sheep than in humans, but that of sheep phosphatidylcholine and phosphatidylethanolamine is lower, mainly because of differing 20:4(n-6) contents.

The above observations show that the tolerance of sheep to a low dietary intake of essential fatty acids does not arise from the replacement of essential fatty acid derived fatty acids by non-essential fatty acid derived entities. This confirms and extends earlier data on the fatty acid composition of brain phosphatidylethanolamine in a number of ruminants (Crawford et al., 1976). Sheep, and probably ruminants in general, have adapted to conserve their essential fatty acid supply for structurally important functions (Reid and Husbands, 1985). Phosphatidylinositol has also

been implicated in a fatty acid salvage pathway (Matsuzawa et al., 1978; Huterer and Wherrett, 1979), but the data do not indicate if the differences between human and sheep phosphatidylinositol reflect adaptive changes in ruminants in general, or just variations between sheep and humans.

CHAPTER IV

LIVER LIPOPIGMENT: LIPIDS AND FLUORESENCE

Introduction

Previous analyses of the lipopigment in the ceroid-lipofuscinoses have been hampered by the small amounts of material isolated from affected brains. The methods used for the purification of the cytosomes were time consuming and complex, and only low yields (milligram quantities) were obtained. (Siakotos et al., 1972; Siakotos and Koppang, 1973; Siakotos and Munkres, 1981; Wolfe et al., 1977; Palo et al., 1982). Since neurones are long-lived cells, lipopigment stored in brain could undergo secondary post-storage changes with time. With neuronal loss pigment would be taken up by macrophages and further modification associated with lysosomal degradation of non-pigment heterophagocytosed material could be expected to occur. Accordingly it was decided to isolate the lipopigment from fresh liver of sheep affected with ceroid-lipofuscinosis, and systematically analyse its composition. Liver was chosen because:

(i) It has a central role in lipid metabolism.
(ii) Its cells undergo mitosis to replace dying cells and consequently there is turnover of pigment cytosomes.
(iii) Liver appears unaffected by the disease, and so there is less likelihood of confounding secondary effects that might occur in brain derived lipopigment.
(iv) It has a relatively simple cell structure, and does not contain myelin, an impurity difficult to remove during lipopigment purification from brain.

Results

(a) Isolation: The method developed for the isolation of liver lipopigment with the use of pronase, yielded granular brown cytosomes with an isopycnic density of 1.18 - 1.20 in sucrose, and two peaks with densities between 1.19 - 1.23 and 1.23 - 1.28 in CsCl. No

similar material was obtained from similarly aged control sheep liver when it was subjected to the same procedures. (Fig. 5).

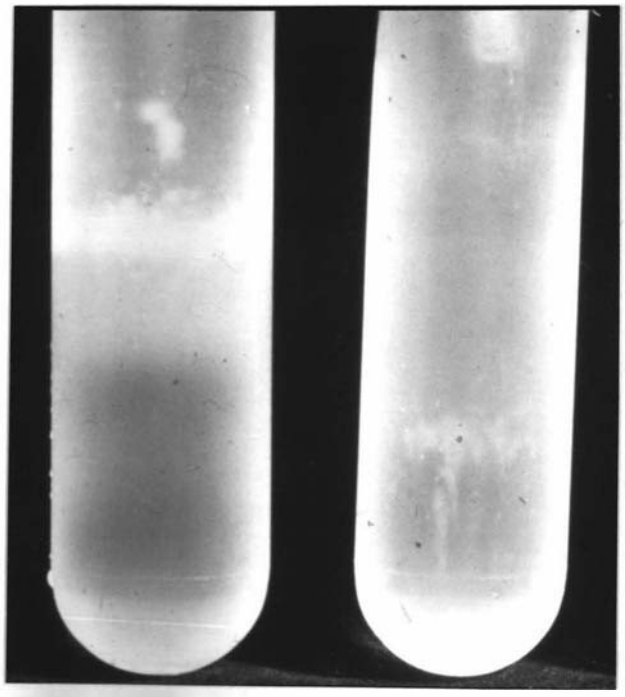


Fig.5. Lipopigment isolation. Lipopigment isolated from affected sheep liver and centrifuged on a CsCl gradient (left), and the result of the same procedure on control liver (right).

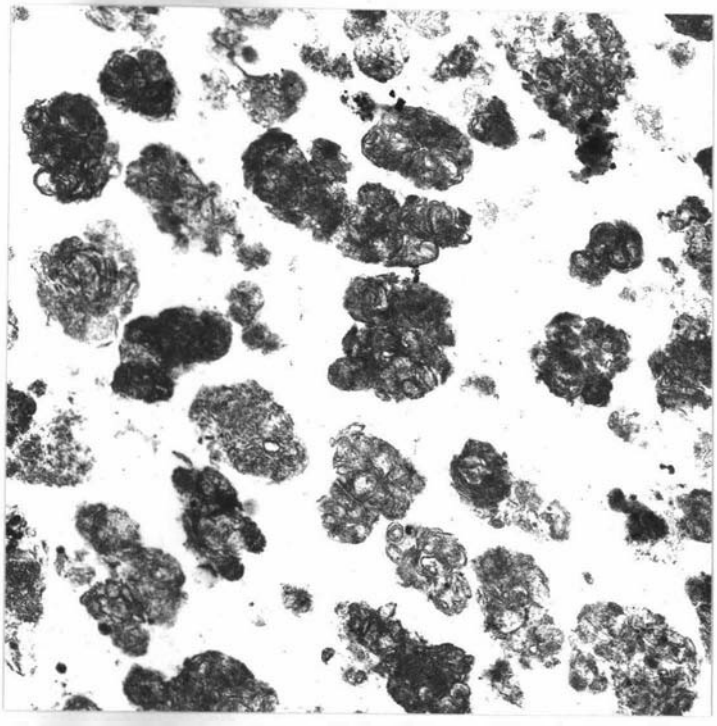


Fig. 6. Isolated lipopigment bodies. Electron micrograph of lipopigment bodies isolated from affected sheep liver with the use of pronase x 35,700.

Isolated lipopigment granules showed an intense yellow-green fluorescence when suspended in glycerol and examined by fluorescence microscopy. Electron microscopic examination of the pellet showed it to be almost entirely composed of lipopigment cytosomes. These had a granular matrix with many and variable membranous profiles (Fig. 6) similar to those observed in electron micrographs of whole tissue. No morphological differences were observed between the cytosomes of different density, nor have any biochemical differences subsequently been established. The yield of lipopigment obtained by this method was high, 2.3 ± 0.39(8) mg of lipopigment lipid being extractable from the lipopigment purified from each g of liver used. This means that at least 8.2% of the total affected liver lipids were from the lipopigment. The isolated lipopigment lipids contained only 51.0% ± 2.16(5) phospholipid compared with the higher value of 73% phospholipid in the total tissue lipids (Table II, Chapter III).

(b) Fluorescence: The excitation and emission spectra of the lipopigment lipids had features in common with those of control and affected total liver lipids. These were excitation maxima at 350 and 370 nm and a broad emission maximum between 490 and 510 nm (Fig. 7). In addition the lipopigment lipid excitation spectrum had a shoulder from 380-470 nm and the emission spectrum a sharp peak at 680 nm. However the lipopigment lipids were less fluorescent than the total tissue lipids, six times the concentration being required to give the same emission intensity.

Variation in the excitation wavelength changed the emission maxima of the lipopigment lipids, indicating that more than one fluorophor was present. This heterogeneity of fluorescence was confirmed by the TLC separation of lipids using sequential development in three solvents. At least 13 fluorophors were found in the lipopigment lipids which also contained an absorbing species (Fig. 8). Some of these fluorophors were present in the total liver lipids albeit in much smaller amounts. Subsequent iodine staining of the plate revealed no material that remained at the origin nor any that had run with the solvent front. Fresh chromatograms of isolated lipopigment phospholipids did not contain any fluorophors except for a yellow and an orange component in the contaminating neutral lipids. However

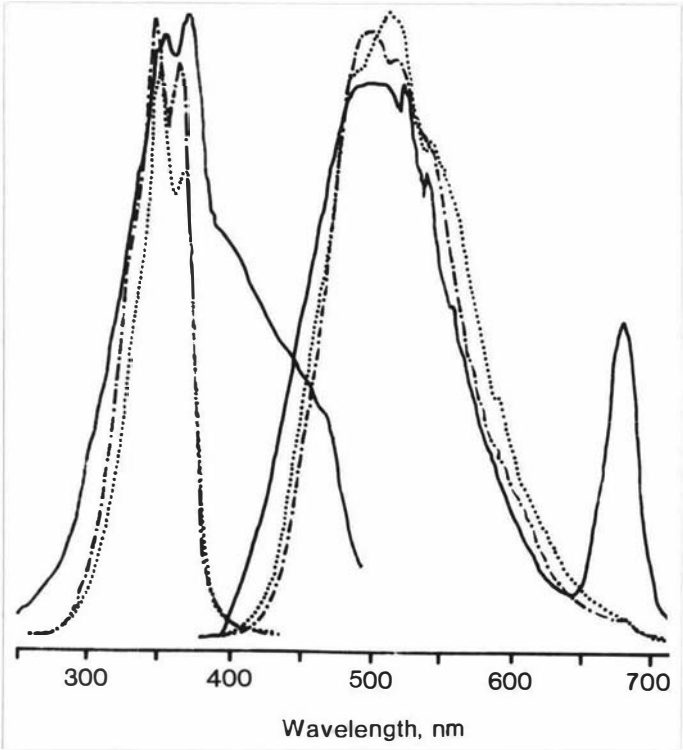


Fig.7. Fluorescence spectra. Spectra recorded at the optimal wavelengths for the lipopigment lipids, viz excitation spectra (left) at 510 nm emission and emission spectra (right) at 370 nm excitation. Lipopigment lipids 1.81mg/ml (solid line). Control liver lipids 0.43mg/ml (broken line). Affected liver lipids 0.31mg/ml (dotted line).

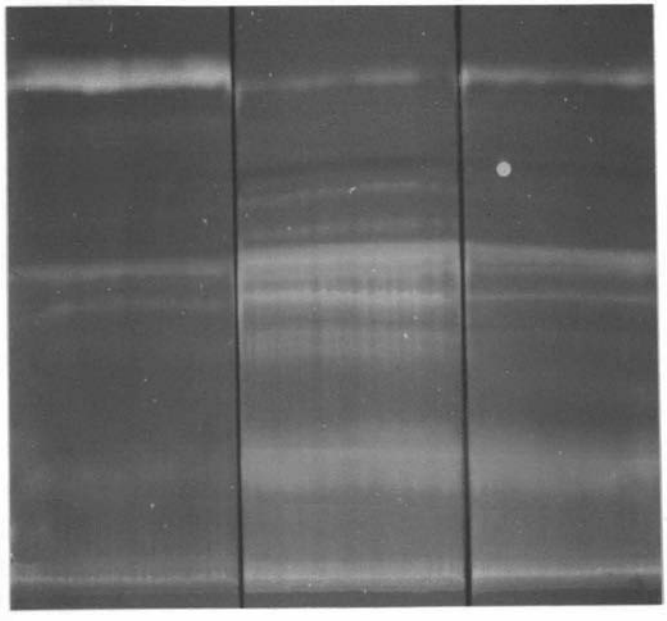


Fig.8. Heterogeneity of lipid fluorescence. The fluorescent components of control liver (left), isolated pigment (middle) and affected liver lipids (right), separated by TLC on a 20x20cm plate developed to 5cm with chloroform:methanol, 1:1; to 10cm with ether:acetic acid, 99:1; and to 20cm with n-hexane:ether:acetic acid; 80:20:1.

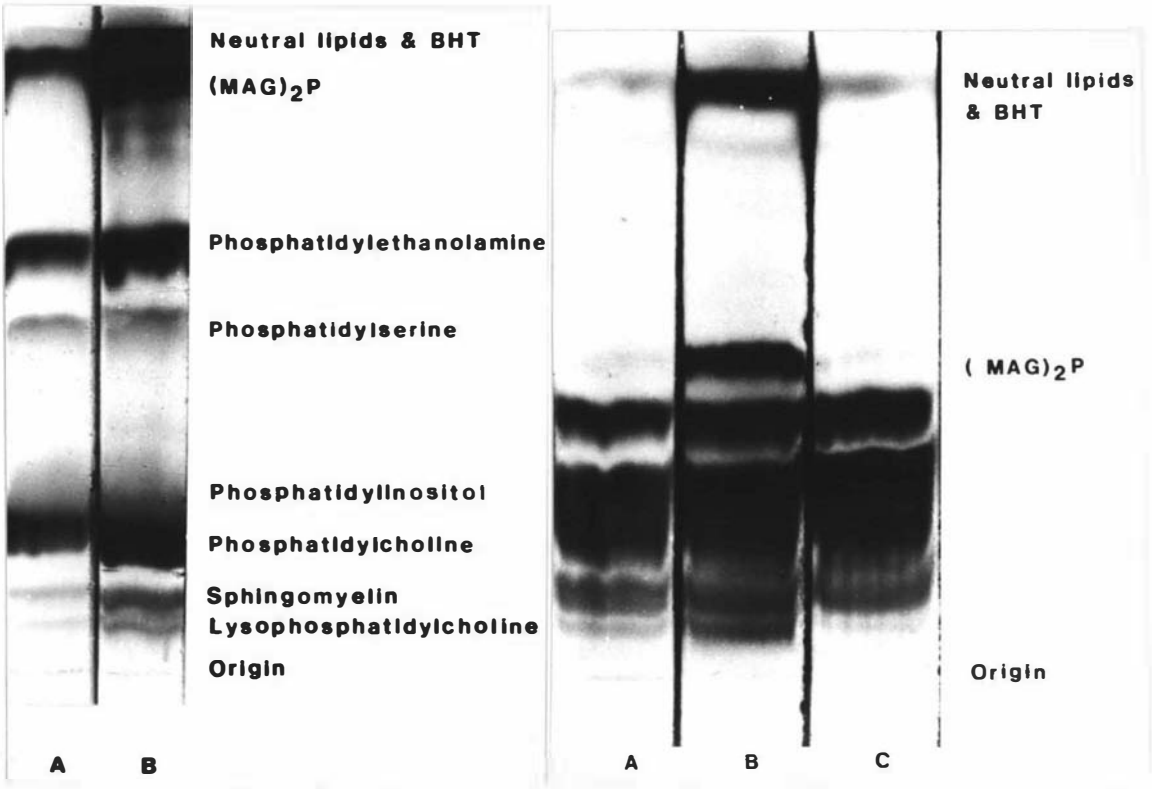
upon standing the TLC plate in air overnight fluorophors appeared, most notably a bright blue fluorescence at the same position as phosphatidylethanolamine.

The lipopigment neutral lipids contained a number of fluorophors, orange, grey, pink, blue and red, that migrated in that order between the origin and cholesterol upon TLC. None of these stained as major components with iodine or were intensely fluorescent. A band identified as ubiquinone absorbed enough light at 366 nm to appear as a dark band. The main contributors to the fluorescence of total affected and control liver lipids were highly fluorescent retinol and retinyl esters.

The upper phase of the lipid extractions of lipopigment bodies exhibited only negligible fluorescence in the spectrofluorometer. The insoluble residue was not fluorescent when directly irradiated with 366 nm light. When suspended as a dry powder in glycerol it was highly fluorescent in the fluorescence microscope, but so were total control and affected tissue residues so treated, and glycerol suspensions of casein and bovine serum albumin.

(c) Phospholipids: TLC of the isolated lipopigment phospholipids showed them to contain the major phospholipid classes, in similar proportions to their occurrence in total liver (Fig. 9). An additional component, that ran near the solvent front in the acidic solvent, migrated as expected for $(MAG)_2P$ in an ammonia containing solvent (Joutti et al., 1976) (Fig.10), and on two dimensional chromatography (Wherret and Huterer, 1972).

(d) Neutral lipids: Lipopigment lipids contained a relatively higher proportion of neutral lipids than either control or affected liver lipids and also a different distribution of individual neutral lipid classes. Free fatty acids, dolichol, ubiquinone and two compounds designated as bands 1 and 2 were major components of the lipopigment lipids (Fig. 3, Chapter III). Retinol and retinyl esters were not present in the lipopigment lipids in measurable amounts. Triglycerides were only a minor component of the lipopigment.



Figs. 9 and 10. TLC of phospholipids. TLC of (A) control liver (B) isolated lipopigment and (C) affected liver phospholipids developed in chloroform:methanol:acetic acid:water; 50:24:4:2 (Fig. 9 left), and chloroform:methanol:ammonia:water; 65:20:2:2: (Fig. 10 right) and stained with iodine.

Lipopigment dolichol was isolated by preparative TLC. Analysis of its chain lengths by reverse phase HPLC showed the same chain length distribution as the porcine dolichol standard. Transmethylation of the compounds identified as bands 1 and 2 yielded dolichol when the reaction products were analysed by TLC and normal phase HPLC. In addition a component migrated as fatty acid methyl esters upon TLC of the transmethylation products of band 1 but no such component was isolated from the transmethylation of band 2.

NMR spectra of bands 1 and 2 showed all the features reported for native dolichol (Breckenridge et al., 1973; Van Dessel et al., 1979), in both the ^{1}H and ^{13}C spectra (Figs. 11 and 12). The additional ^{1}H resonance at 1.25 ppm and ^{13}C resonance at 29.7 ppm, that were relatively larger in band 1 than band 2 were attributed to $-(CH_2)_n-$ and the increase in the 0.90 ppm ^{1}H resonance to additional terminal methyl groups of alkyl chains. The ^{1}H resonance at 4.05 ppm in band 2 was attributed to a primary alcohol ester.

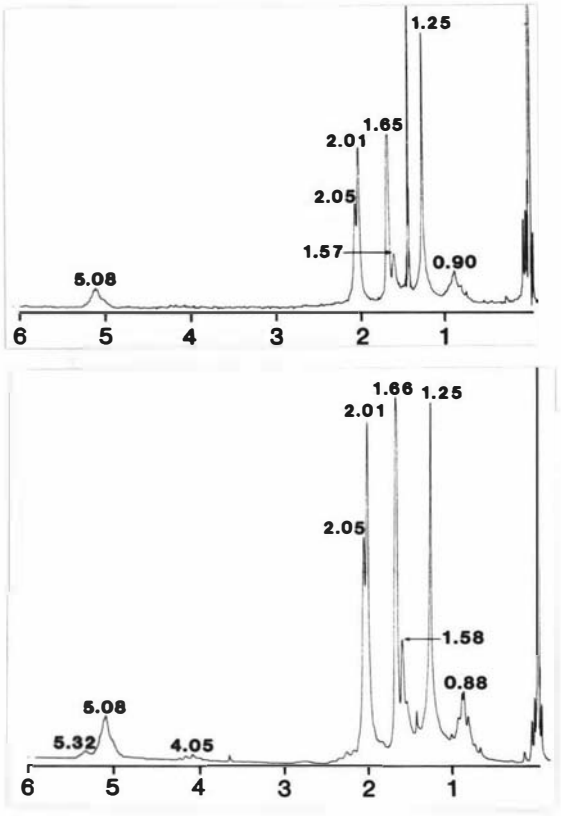


Fig.11. ^{1}H NMR spectra. Spectra of band 1 (top), and band 2 (bottom). Chemical shifts are in ppm from TMS. The peak at 1.40ppm arises from residual cyclohexane.

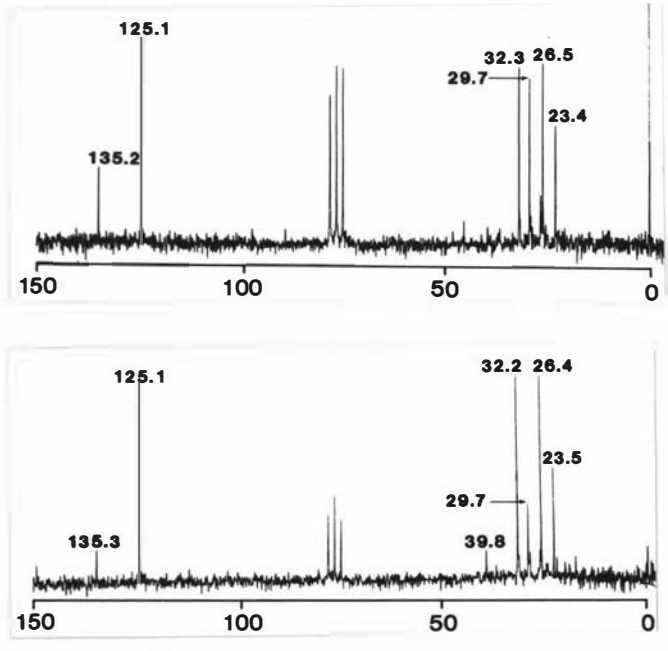


Fig.12. ^{13}C NMR Spectra. Spectra of band 1 (top), and band 2 (bottom). Chemical shifts are in ppm from TMS. The three peaks at approximately 78 ppm arise from $CDCl_3$.

Resonances at 5.32, 2.05 and 1.58 ppm in the ^{1}H spectra, and at 39.8 ppm in the ^{13}C spectrum of band 2 were attributed to trans isoprenoid units in the dolichol chain.

(e) Fatty acid compositions: The fatty acid profiles of the major phospholipids and the free fatty acids of the isolated lipopigment were determined by gas-liquid chromatography (Table 6). No large differences were observed between those of the lipopigment phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine. Those of phosphatidylcholine and phosphatidylethanolamine were similar to those of these lipids in the total tissue (Table 4), except for more of the linoleate derived species. Phosphatidylcholine contained correspondingly less of the linolenate derived species and phosphatidylethanolamine less of the saturated fatty acids. The major fatty acids of $(MAG)_2P$ were linoleate, 42.9%, and linolenate, 16.5%. Saturated fatty acids and 20 and 22 carbon entities were present in this compound in very reduced amounts.

The free fatty acids in the lipopigment lipids contained the full range of medium and longer chain fatty acids found in lipopigment phospholipids. They contained a greater proportion of 18:1(n-9), and fatty acids less than 16 carbons long, than any of the phospholipids.

(f) Non-lipid components : Elemental analysis of the insoluble residue remaining after the extraction of lipopigment lipids showed it to contain 53.19% C, 7.81% H, 12.71% N and 2.59% S by weight, consistent with it being protein. Gravimetric analysis showed it to be 70 ± 5.7% (n = 4) of the total lipopigment mass, and Lowry protein estimations of sonicated suspensions of lipopigment bodies gave the same proportion as protein.

Discussion

The method described here allowed the isolation of lipopigment cytosomes from affected sheep liver in quantities sufficient for a systematic analysis of their chemical constituents for the first time. Isolated lipopigment cytosomes retained the characteristic morphology and fluorescent properties that they exhibited in situ.
These properties and their isopycnic densities in both CsCl and

Table 6
Isolated lipopigment phospholipid fatty acids

Mean percentage fatty acids by weight of various phospholipids from lipopigment isolated from livers of four sheep affected with ceroid-lipofuscinosis. To facilitate presentation of results standard errors were omitted but within each column means and standard errors were positively related. ECL's are the mean value of 24 determinations. Standard errors for these ranged from 0.013 for 17:0 to 0.071 for 22:6(n-3) fatty acids.

Fatty acid	ECL	Phosphatidyl-choline	Phosphatidyl-inositol	Phosphatidyl-serine	Phosphatidyl-ethanolamine	$(MAG)_2P$	Free fatty acids
16:0	defined	14.3	5.6	5.9	4.6	3.0	14.8
16:1s	16.54	1.3	.6	.8	.5	2.6	2.9
17:0	17.01	1.3	1.5	1.9	1.1	3.1	1.4
17:1s	17.56	.6	.4	.5	.4	.8	1.1
18:0	defined	23.1	27.9	36.1	25.5	4.8	10.5
18:1(n-9)	18.44	20.7	16.4	14.9	16.6	12.4	30.2
18:1(n-5)	18.84	.6	.1	.6	.3	.1	.1
19:0	19.02	.3	.8	.2	.3	.5	.6
18:2(n-6)	19.26	8.9	5.2	5.1	7.8	42.9	9.7
18:3(n-3)	20.16	2.6	1.7	1.6	2.2	16.5	4.3
20:1(n-9)	20.42	2.2	1.0	.9	1.8	.1	1.6
20:2(n-6)	21.26	.4	.3	.4	.6	.3	.4
20:3(n-9)	21.38	.4	1.9	2.7	.4	.1	.7
20:3(n-6)	21.71	.6	.9	1.2	.5	1.0	.6
20:4(n-6)	22.07	5.6	9.1	10.0	14.3	3.0	6.1
20:5(n-3)	22.98	3.6	3.9	4.4	4.7	1.1	2.4
22:4(n-6)	24.8	.5	4.0	1.4	.3	.1	.1
22:5(n-3)	24.94	6.3	8.4	6.8	9.3	2.2	3.2
22:6(n-3)	25.28	5.2	7.9	3.6	8.2	2.3	3.2
Total polyunsaturated		34.1	43.3	37.2	48.3	69.3	30.6

sucrose were similar to those of lipopigment isolated from brains of humans affected with ceroid-lipofuscinosis (Siakotos et al., 1972; Wolfe et al., 1977). Except for the double excitation peak, the fluorescence excitation spectrum of the liver lipopigment lipids (Fig. 7) was similar to that of the lipids from affected human and sheep brain lipopigment (Jolly et al., 1980; Wolfe et al., 1977; Siakotos and Koppang, 1973), but the emission spectrum maximum was at 60-70 nm longer wavelength and the emission spectrum contained an additional previously unreported peak at 680 nm. However despite the highly fluorescent nature of the lipopigment bodies the lipopigment lipids were only one sixth as fluorescent as total liver lipids. As a consequence the distinctive features of the lipopigment lipids, the 680 nm emission peak and the excitation shoulder, were barely discernible in the affected liver lipids spectra even though these contained at least 8.2% lipopigment lipids.

TLC showed that the lipopigment lipids contained a number of fluorophors, none of which were major lipid components. Similar heterogeneity of fluorescent components has also been reported in lipids of affected human brains (Garg et al., 1981). The liver lipopigment lipids contained no fluorescent component that could equate with a Schiff base polymer consequent to lipid peroxidation as found and interpreted in human brain lipopigment lipids by other workers (Siakotos et al., 1972). No fast migrating fluorescent material that could equate with the postulated "polymalonaldehyde" (Gutteridge et al., 1982b) was found either. Although it did not fluoresce as a solid mass the anhydrous proteinaceous residue of lipid extracted bodies was highly fluorescent when suspended in glycerol. However so were the delipidated proteins of the total affected and control tissues and suspensions of the proteins casein and albumin. The fluorescence of the lipopigment bodies in situ is likely therefore to arise from the environmentally dependent fluorescence of protein, in a similar way to the observed fluorescence of the globules of protein that accumulate in the livers of people with alpha-1-antitrypsin (alpha-1-protease inhibitor) deficiency (De Lellis et al., 1972). This fluorescence may be augmented by the cumulative fluorescence and interaction of the lipid fluorophors but the fact that none of these are intense fluorophors or present in other than small amounts militates against them being of pathogenic significance.

The isolated liver lipopigment contained a similar proportion of phospholipid species to those of lipopigment isolated from affected brains (Siakotos and Koppang, 1973; Wolfe et al., 1981). As with brain lipopigment these included $(MAG)_2P$ at levels much higher than observed in the total tissue (Siakotos et al., 1972). This compound is a normal lysosomal lipid component (Bleistein et al., 1980), and its level is further elevated in secondary lysosomes that arise from a variety of induced and inherited diseases (Stremmel and Debuch, 1976; Tjiong et al., 1978; Nakashima et al., 1984). In some of the induced diseases of rats, it contains high levels of polyenoic fatty acids, the most notable species often being 22:6(n-3). In the affected sheep liver lipopigment it also has a high content of polyunsaturated fatty acids but these are mainly the 18 carbon essential fatty acids (Table 6). As the metabolism of essential fatty acids is different in sheep to rats (Reid and Husbands, 1985), this difference is likely to be between species rather than specific to the ceroid-lipofuscinoses. As such it may be a consequence of the postulated involvement of $(MAG)_2P$ in salvaging fatty acids from other complex organelles during disassembly in lysosomes (Huterer and Wherrett, 1979). It is of interest that phosphatidylinositol is implicated as an acyl transfer species in this process, and that the fatty acid profile of this phospholipid is significantly different in sheep and human brains. (Chapter III).

The fatty acid profiles of the other four lipopigment phospholipids examined closely resemble those reported for these phospholipids in isolated rat liver lysosomes (Henning and Heidrich, 1974), except for lower levels of 16:0 and correspondingly higher levels of 18:1(n-9). There was no indication of a depletion of any polyunsaturated fatty acids in any of these phospholipids, nor in the lipopigment free fatty acids (Table 6), which would be expected if there had been significant abnormal peroxidation of polyunsaturated fatty acids. Together with the analyses of the fatty acids in the total tissue lipids (Table 4, Chapter III), these results indicate that there is no abnormality in phospholipid or polyunsaturated fatty acid metabolism in this disease. As such they indicate that the previously observed differences in sheep brain grey matter phosphatidylethanolamine fatty acid profiles (Table 5, Chapter III) are probably secondary to brain atrophy.

Neutral lipids accounted for approximately 50% of the liver lipopigment lipids, as they do in lipopigment isolated from affected human brain (Wolfe et al., 1981). Major components of these neutral lipids were cholesterol, free fatty acids, dolichol, ubiquinone and the compounds designated as bands 1 and 2. On the basis of their chemistry and NMR spectra (Figs 11 and 12) bands 1 and 2 were assigned as dolichyl esters, band 1 being those esters with distinctively longer chain acid moieties. A difference in TLC migration of such dolichyl esters has been reported (Steen et al., 1984). As dolichol dolichoate also migrates in this region its presence in the lipopigment lipids cannot be excluded.

Dolichol has previously been reported as a major component of the lipopigment from brains of humans affected with the infantile, late infantile and juvenile forms of ceroid-lipofuscinosis and it is measurably enriched in the whole brains of these patients. However it also accumulates in normal brains with age and in brains of patients affected with Alzheimer's disease (Ng Ying Kin et al., 1983). Lysosomes contain the major pool of cellular dolichol (Wong et al., 1982; Henning and Stoffel, 1972). The presence of these neutral lipids and $(MAG)_2P$ in the lipopigment thus support a lysosomal involvement in lipopigment biogenesis. Together with the lack of any discerned differences in the total tissue neutral lipid values, these results indicate no specific defect in neutral lipid metabolism.

The major component of the lipopigment bodies was the insoluble residue remaining after lipid extraction. Elemental analysis and protein determinations indicated that this was protein, rather than a retinoid complex or the consequence of lipid peroxidation. Further analysis of this material was therefore indicated. A method of isolating lipopigment bodies without the use of pronase was required to ensure the integrity of isolated lipopigment polypetides. Such a method is presented in the following chapter, together with the results of subsequent studies of the lipopigment proteins.

CHAPTER V

LIPOPIGMENT PROTEINS, DOLICHOL AND UBIQUINONE

Introduction

In the previous chapter the fluorescence, phospholipids, fatty acids and neutral lipids of lipopigment isolated from affected sheep livers were examined. Lipids composed less than 25% of the lipopigment mass and there was no evidence of a disturbance of lipid metabolism or of abnormal lipid peroxidation. The presence of the lipids bis(monoacylglyceryl)phosphate [$(MAG)_2P$], dolichol, dolichyl esters and ubiquinone in the lipopigment strongly suggested a lysosomal derivation of the lipopigment cytosomes. Most of the lipopigment was proteinaceous. To analyse this material unambiguously, methods of isolating lipopigment without the use of the proteolytic enzyme pronase were required, otherwise the possibility of proteolytic cleavage of lipopigment polypeptides during isolation would make any results unsound. There is also the possibility that the accumulation of proteinaceous material is part of a general accumulation of lysosomal membrane, rather than the specific accumulation of protein. Dolichol and ubiquinone are markers for lysosomal membranes (Wong et al., 1982; Henning and Stoffel, 1972). Their concentrations in the lipopigment, relative to protein, would indicate which of these mechanisms is involved in lipopigment biogenesis. It was also desirable to compare lipopigment from a number of tissues so that any tissue specific phenomena could be recognised.

Results

(a) Isolation: Initial experiments on the isolation of lipopigment without pronase were carried out on liver biopsy samples, 0.5-2g. Highly purified lipopigment bodies were isolated by a simple combination of osmotic lysis, sonication and differential centrifugation. The protocol developed was suitable for much larger amounts of tissue, and with small modifications the method was applicable to other tissues. Using these protocols (see Chapter II) large amounts of purified lipopigment were obtained from liver,

kidney, pancreas and brain of affected sheep in much shorter times than by any previously reported procedures. Lipopigment cytosomes from all tissues had isopycnic densities in CsCl of over 1.19 (Table 7). Liver lipopigment had a bimodal density profile. However no morphological differences were observed between the two populations.

Table 7

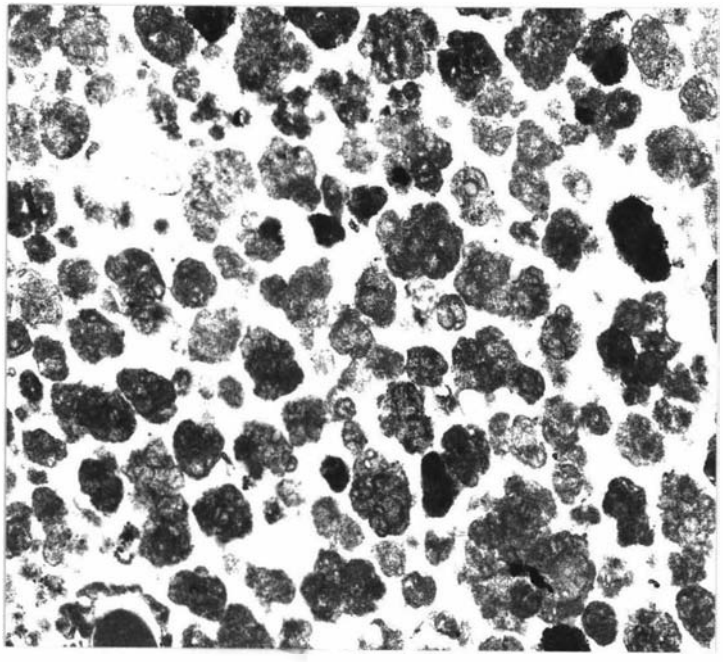
Lipopigment densities

Mean isopycnic densities in CsCl gradients of lipopigment isolated from two sheep affected with ceroid-lipofuscinosis.

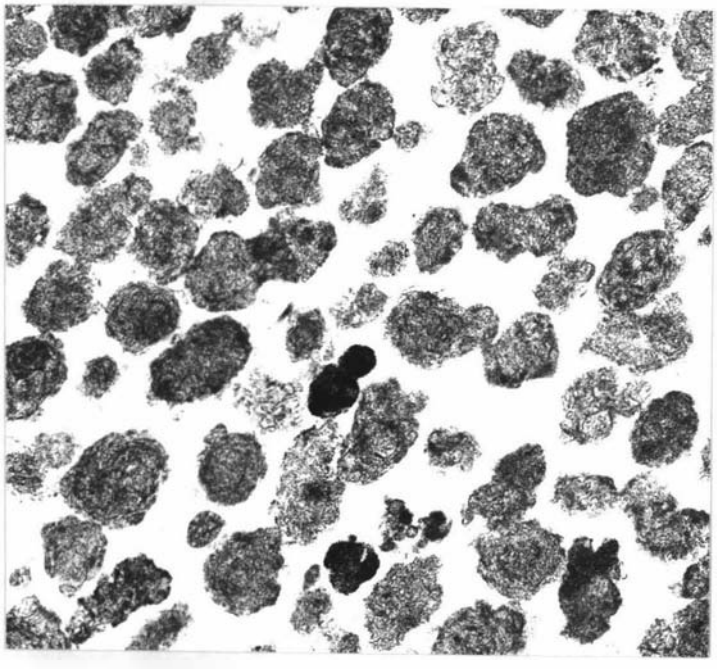
Tissue	Density
Liver	1.19 - 1.23 and 1.23 - 1.28
Kidney	1.21 - 1.26
Pancreas	1.19 - 1.22
Brain	1.19 - 1.24

The lipopigment bodies obtained from each tissue had electron dense membranous structures typical of bodies in situ in the parent organs (Fig. 13). They displayed an intense green to yellow-green fluorescence by fluorescence microscopy. Isolated lipopigment from liver was dark brown, from kidney a mid-brown, from pancreas tan, and a multilayered pellet was obtained from brain that contained material ranging in colour from tan to almost black.

The yield of lipopigment bodies isolated, per g wet weight of total tissue, was similar for all four tissues (Table 8) and did not appear to be age dependent.

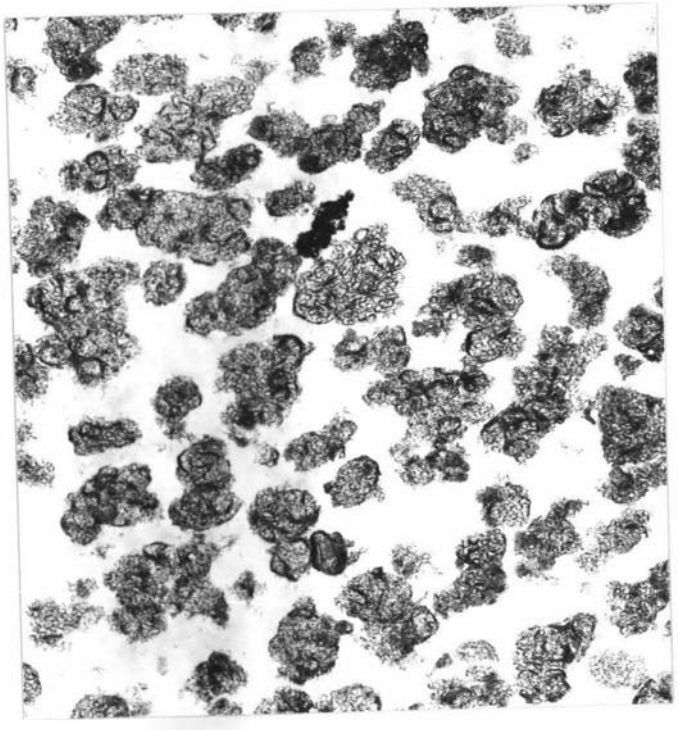


A

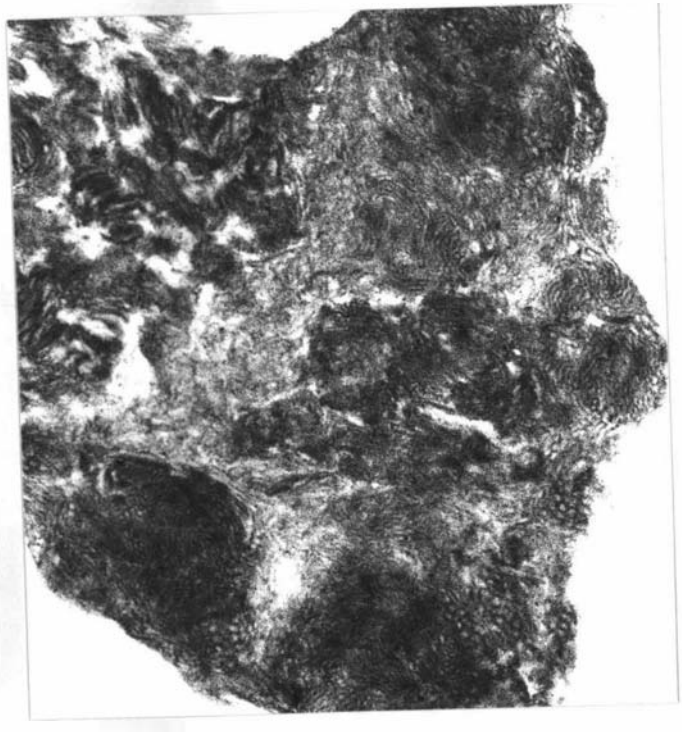


B

Fig. 13. Morphology of lipopigment bodies. Electron micrographs of lipopigment bodies isolated without the use of pronase. (A) from liver, x 11,200; (B) from kidney, x 11,200.



C



D

Fig. 13.(continued) Morphology of lipopigment bodies. (C) from pancreas, x 11,200 and (D) from brain, x 64,100.

Table 8
The yield, and protein and lipid proportions of isolated lipopigment bodies

The yield of lipopigment bodies isolated from liver, kidney, pancreas and brain, in mg dry mass of lipopigment/g wet weight of tissue used for their isolation, and the % of the total dry mass of lipopigment that is protein and lipid, ± s.e.m. with the number of experiments in parenthesis.

Tissue	Yield	% Protein	% Lipid
Liver	1.4 ± 0.65 (4)	73.6 ± 6.35 (4)[a]	16.8 ± 3.98 (4)
		65.4 ± 6.39 (4)[b]	
		74.8 (1)[c]	
Kidney	1.3 ± 0.26 (4)	66.1 ± 2.74 (6)[a]	27.1 ± 3.50 (4)
		67.2 ± 4.18 (4)[b]	
		73.9 (1)[c]	
Pancreas	1.5 ± 0.38 (5)	72.2 ± 2.55 (5)[a]	20.9 ± 3.98 (5)
		70.0 ± 2.38 (5)[b]	
Brain	1.4 ± 0.16 (3)	66.2 ± 3.05 (3)[a]	15.8 ± 4.99 (4)
		60.3 ± 4.65 (3)[b]	

[a] Dry weight of the insoluble residues after lipid extractions

[b] Protein as estimated by the method of Lowry et al.(1951).

[c] Hydrolysable amino acid units of delipidated lipopigment.

(b) Lipopigment composition: The insoluble residues formed during lipid extractions were the major component of lipopigment bodies isolated from all four tissues and accounted for at least two-thirds of the lipopigment mass (Table 8). No significant differences ($P > 0.05$, Student's t test) were found in the proportion of this material in the lipopigment bodies from any of the tissues. Similar proportions of the lipopigment mass were assayed as protein, using bovine serum albumin as a standard. Quantitative amino acid analyses of hydrolysed delipidated lipopigment bodies from liver and kidney were also in agreement with these values.

Most of the remainder of the lipopigment mass was lipid, measured as between 16-27% of the lipopigment isolated from the various tissues. These apparent variations in the proportion of lipid in the various lipopigments were not significant however. Lipid and protein together accounted for over 90% of the lipopigment from liver, kidney and pancreas, and 82% of that isolated from brain.

(c) Lipopigment proteins: Lipopigment bodies from all tissues proved difficult to solubilize and disaggregate. A large range of possible solvents were tried including 8M urea, 6M guanidine-HCl, 1% trifluoroacetic acid, 70% formic acid, 30% pyridine, concentrated ammonia, 0.1M ammonium bicarbonate with 8M urea, 70 and 100% dimethylsulphoxide, 70 and 100% acetonitrite, 10mM EDTA, 4M NaCl and the following detergents at 1%; chromotropic acid, Brij35, cetylpyridinium chloride, tyloxapol, nonidet-P40, deoxycholic acid, n-tetradecyl-N,N dimethylammino-3-propanesulphonate, and SDS, all with and without the addition of 5% 2-mercaptoethanol. Of these only 70% formic acid and 1% SDS in the presence of 5% 2-mercaptoethanol dissolved the lipopigment cytosomes or delipidated protein obtained from them. The latter allowed SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the lipopigment proteins. Initial experiments with the loadings normally used for Coomassie blue staining resulted in a smear running the length of the gel. At the lower loadings required for silver staining, and by using the gentle disaggregation techniques described in Chapter II, discrete bands were obtained (Fig. 14).

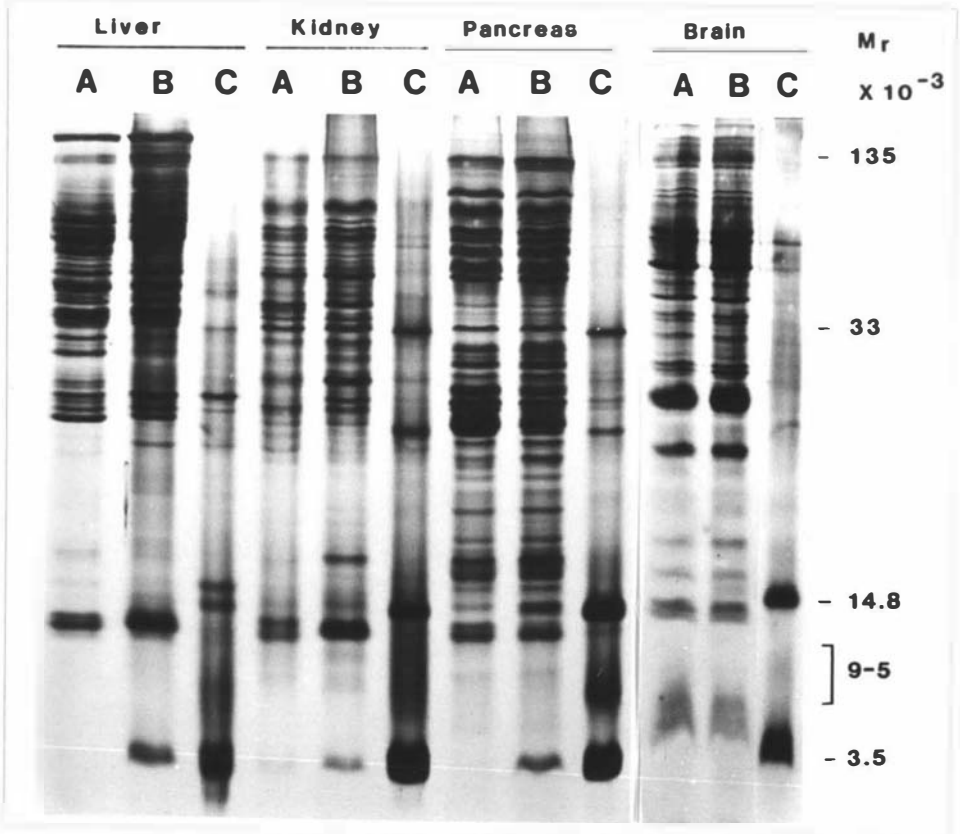


Fig. 14. SDS-PAGE of **lipopigment bodies and total** tissue homogenate. Silver staining of isolated lipopigment and total control and affected tissue homogenates. Lanes A, control tissue homogenates; Lanes B, affected tissue homogenates; Lanes C, isolated lipopigment bodies. Molecular weights (M_r) were calibrated from the positions of standards in other gels.

Lipopigment bodies from all four organs were characterised by two heavily staining bands, one that ran to the lower region of the gel, and which had an apparent M_r of 3,500 and another with an M_r of 14,800. Between these two bands was a diffuse staining area, in the M_r range of 5,000 - 9,000. As the band with an apparent M_r of 3,500 occurred at the limit of resolution in the gel it may also contain material of lower molecular weight. SDS-PAGE of the insoluble residue left after the extraction of isolated liver lipopigment lipids gave the same pattern as that obtained with the intact lipopigment. Lipopigment from all tissues contained a number of minor components of higher molecular weight. Lipopigment from liver, kidney and pancreas also contained a number of higher molecular weight proteins that stained with intermediate intensity, but unlike the lower molecular weight proteins, none of these were common to lipopigment from all four tissues.

SDS-PAGE of homogenates of whole liver and whole pancreas from affected sheep clearly showed the 3,500 M_r band whereas this was not present in homogenates of these organs from normal control animals. A small amount of low molecular weight material in homogenates of normal kidney made this distinction between normal and affected homogenates slightly less obvious. The 3,500 M_r material was not seen in homogenates of either affected or normal brains. A slight enrichment in the 14,800 M_r band was observed in total homogenates of all four affected tissues when these were compared with control tissue homogenates (Fig. 14).

Some preparations were carried out in the presence of lysosomal protease inhibitors, since lysosomal proteases released during the isolation of the lipopigment might cleave larger proteins into the low M_r components observed above. Serine and thiol proteases were inhibited with phenylmethanesulphonylfluoride and iodoacetate respectively, and metal dependent proteases with EDTA. Mercaptoethanol was omitted to avoid possible affects of thiol activated proteases (see Chapter II). None of these procedures alone or in combination made any difference to the pattern of bands obtained upon SDS-PAGE of the isolated lipopigment.

The low M_r components of the lipopigment bodies were not as sensitive to Coomassie blue staining, amido-black staining or to the Bio-Rad silver stain alone as they were to the double silver stain with a diethyldithiocarbamate wash described here, nor was the difference between control and affected total tissue homogenates as apparent with these other stains. None of the lipopigment material from any tissue stained for RNA after SDS-PAGE. The carbohydrate stain gave a faint reaction with the diffuse low molecular weight material in total brain homogenates, and a very faint reaction with the 14,800 M_r and the 3,500 M_r band in the brain lipopigment bodies. No carbohydrate staining was observed upon SDS-PAGE of any of the lipopigments from other tissues or total tissue homogenates.

In order to ensure that the staining of the low M_r material arose from it being protein and not from the staining of non-protein components of the lipopigment, lipopigment bodies were incubated with a nuclease

free protease and the resultant digest examined by SDS-PAGE. All the bands from liver, kidney, brain and pancreas lipopigment bodies were degraded with the formation of a diffuse staining area at a lower M_r than observed previously for any component, and a general light background smear (Fig. 15).

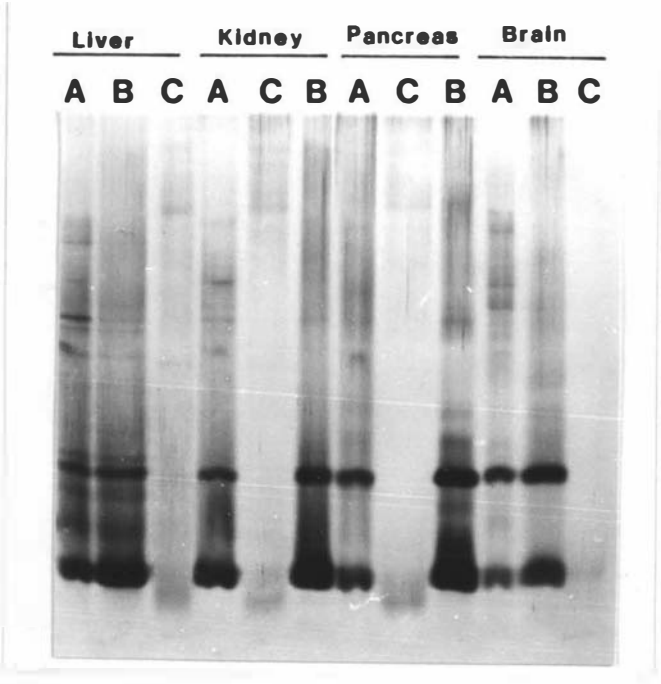


Fig.15. Protease digestions of lipopigment bodies. Silver stained SDS-PAGE of nuclease free protease digestions of lipopigment bodies isolated from liver, kidney, pancreas and brain. Lanes A, unincubated lipopigment bodies (10µg protein/lane); Lanes B, 5mg/ml of lipopigment bodies dissolved in SDS incubated at 37°C for 195 min (30µg protein/lane); Lanes C, 5mg/ml of lipopigment bodies dissolved in SDS and incubated with 1mg/ml of nuclease free protease at 37°C for 195 min (30 µg lipopigment protein/lane).

(d) Lipopigment isoprenoids: The levels of cholesterol, dolichol and ubiquinone in the lipopigment bodies from the four tissues are compared in Table 9. Significant differences in the levels of these isoprenoids in the lipopigment from the various tissues were observed. The highest level of dolichol was found in pancreas derived lipopigment, of cholesterol in brain derived material and the lowest level of ubiquinone in that from liver.

Table 9
Cholesterol, dolichol and ubiquinone levels in lipopigment bodies

The amount of cholesterol, dolichol and ubiquinone in lipopigment bodies isolated from liver, kidney, pancreas and brain. Values are ug/mg dry mass of lipopigment bodies ± s.e.m. with the number of experiments in parenthesis.

Tissue	Cholesterol	Dolichol	Ubiquinone
Liver	3.6 ± 0.63 (3)	10.7 ± 3.17 (3)	0.7 ± 0.28 (3)
Kidney	7.7[a]± 1.09 (4)	9.9 ± 0.87 (4)	1.9[a]± 0.33 (4)
Pancreas	7.5 ± 1.97 (6)	20.8[c]± 2.17 (6)	1.8 ± 0.98 (6)
Brain	28.3[b]± 6.18 (4)	8.8 ± 1.18 (4)	1.9[a]± 0.38 (4)

[a] significantly different to the liver value $P \leq 0.05$
[b] significantly different to the liver value $P \leq 0.01$
[c] significantly different to the liver, kidney and brain values $P \leq 0.001$.

Discussion

Simple rapid methods for the isolation of lipopigment from several tissues of sheep affected with ceroid-lipofuscinosis, without the use of the proteolytic enzyme pronase, have allowed the characterisation of the lipopigment proteins. Analyses of the purified lipopigment showed that the high proteinaceous content of the lipopigment from liver, as described in the previous chapter, is a property of the lipopigment from other tissues as well (Table 8). Most of the remainder of the lipopigment mass from all the tissues studied was lipid.

Dolichol has previously been shown to be a major component of neutral lipids of lipopigment bodies isolated from the brains of humans affected with neuronal ceroid-lipofuscinosis (Ng Ying Kin, et al., 1983) at similar levels to those reported here for the sheep derived lipopigments (Table 9). Lysosomes are the subcellular organelles that contain most intracellular dolichol (Keller et al., 1982). The dolichol contents of lipopigment from liver, kidney and brain of sheep with ceroid-lipofuscinosis, respectively 14.5, 15.0 and 13.3 µg/mg of protein (calculated from data in Tables 7 and 8), are also very similar to the dolichol content reported in a lysosome enriched rat liver fraction, 15.1 µg/mg of protein (Wong et al., 1982). Because of the difficulty in isolating pure lysosomes, and potential losses during saponification (Pullarkat and Reha, 1982), this latter value may be an underestimate. The dolichol contents of the isolated lipopigment from all four tissues examined were considerably lower than that reported for normal human brain grey matter lipofuscin (Pullarkat and Reha, 1982).

The levels of cholesterol and ubiquinone in normal rat liver lysosomal membranes, 45.6 and 8.1 µg/mg of protein respectively (Henning and Heidrich, 1974; Henning and Stoffel, 1972), are approximately eight times the levels found in the purified liver lipopigment, 4.9 and 1.0 µg/mg of protein respectively (calculated from the data in Tables 7 and 8). Only the cholesterol content of lipopigment isolated from brain approaches the normal liver lysosomal membrane value. Thus although these three isoprenoids are major components of the lipopigment lipids, they are not specifically enriched above the

levels expected for lysosome derived organelles. Their levels are mainly lower than expected if the lipopigment cytosomes were merely the aggregation of lysosomal membranes, indicating the specific accumulation of protein, the other major component of the lipopigment. The variable levels of cholesterol, dolichol and ubiquinone in the lipopigment bodies from the different organs (Table 9) may however contribute to the differences in morphology of these isolated bodies (Fig. 13), differences that are also observed in situ (Jolly et al., 1980; Jolly et al., 1982).

The proteins of the isolated lipopigment bodies are predominantly low molecular weight proteins and these are the common feature of the proteins of the lipopigment from all four tissues examined (Fig. 14). They are not fragments of larger proteins cleaved by proteases released and/or activated during the isolation of the lipopigment bodies, otherwise the inhibition of potential lysosomal proteases during the isolation of the liver lipopigment would have changed the pattern of liver lipopigment proteins.

Low M_r proteins are not normal components of primary, or Triton WR-1339 or dextran induced secondary lysosomal membranes. These are characterised by a large number of proteins in the 60,000 - 100,000 M_r range, some of which are glycoproteins (Yamamoto et al., 1980; Burnside and Schneider, 1982). Small molecular weight proteins were not observed in these membranes (Burnside and Schneider, 1982). Conversely the SDS-PAGE patterns of the isolated lipopigments (Fig. 14) show that normal lysosomal proteins can only be minor components of the lipopigment bodies. Thus, unlike the major lipopigment lipids, the major lipopigment proteins are not those of normal lysosomal membranes.

The extent of heterogeneity within the low M_r lipopigment proteins is not clear. The band at 14,800 Mr appears homogeneous. The material between 5,000 and 9,000 M_r is certainly heterogeneous. As the 3,500 M_r material runs at the bottom of the gel it is impossible to judge the extent of heterogeneity in it. All of these components could also be subunits of higher molecular weight aggregates or proteins. The requirement for gentle disaggregation techniques prior to SDS-PAGE, rather than the usual boiling in 2-mercaptoethanol is not

unique to the lipopigment. Other hydrophobic proteins also aggregate randomly when boiled with 2-mercaptoethanol in SDS, prohibiting SDS-PAGE analysis (Teather et al., 1978; Stoker et al., 1983; Ito, 1984).

The particular sensitivity of the 14,800 M_r and 3,500 M_r lipopigment bands to silver staining after treatment with diethyldithiocarbamate indicates an as yet undetermined common distinctive feature of these components. Differential sensitivity of proteins to different silver staining protocols has previously been noted (Friedman, 1982) but no general theory has yet emerged that explains these observations.

Whatever the nature of the low M_r lipopigment proteins they are the major component of the lipopigment bodies, and are the only molecular species so far detected in the ceroid-lipofuscinoses enriched to the extent that affected total tissues can be distinguished from normal tissues by their presence. Their presence is therefore likely to be a direct consequence of the genetic lesion in this form of ceroid-lipofuscinosis.

CHAPTER VI

THE ROLE OF METALS IN LIPOPIGMENT FORMATION

Introduction

The concentration of iron in cerebrospinal fluid (CSF) of patients affected with ceroid-lipofuscinosis has been reported as higher than that in controls (Gutteridge et al., 1982a; Johansson et al., 1984) and has been associated with apparently high lipopigment iron and copper contents (Siakotos and Koppang, 1973; Johansson et al., 1984). These metals have recently been considered to be catalysts necessary for the conversion of lipid peroxides to the fluorophors postulated as characteristic of the lipopigment (Gutteridge, 1984; 1985). Based on these findings a general defect in iron metabolism has been advanced as underlying the pathogenesis of the ceroid-lipofuscinoses (Gutteridge et al., 1983). Desferrioxamine, a chelator of free iron, has consequently been added to the "anti-oxidant therapy" given to some patients with ceroid-lipofuscinosis, (Westermarck and Santavuori, 1984), but desferrioxamine can have serious harmful side-effects (Halliwell et al., 1985; Halliwell and Gutteridge, 1986). It was therefore thought pertinent to check the generality of this proposed role of copper and/or iron in the pathogenesis of ceroid-lipofuscinoses. The metal contents of CSF and other tissues of affected sheep were therefore analysed, and compared with control values and the metal contents of lipopigment isolated from affected tissues.

Results

(a) Metals in liver, kidney, pancreas, and isolated lipopigments: Metals contributed 1.7% of the dry mass of liver and pancreas derived lipopigment, 1.3% of kidney lipopigment and 1% of the lipopigment in brain (Table 10). Major contributors were copper, iron, calcium, zinc, magnesium, sodium and potassium. Small amounts of molybdenum, strontium, manganese, aluminium, nickel and chromium were also detected. Selenium, cadmium, cobalt and tin were assayed for but were not present in amounts that allowed quantitation by the method used.

Table 10
The metal content of lipopigment isolated from sheep affected with ceroid-lipofuscinosis

Values are µg/gm dry tissue ± s.e.m. Isolated lipopigment data arise from 8 animals for liver and 4 animals for the other tissues. Total affected values arise from 4 animals and total control data from 10 animals.

Metal		Tissue			
		Liver	Kidney	Pancreas	Brain
Cu	Isolated lipopigment	12715 ± 1774	136 ± 27	39 ± 9	451 ± 200
	Total affected	905 ± 219	16 ± 5	5 ± 1.5	24 ± 4.9
	Total control	465 ± 29	17 ± 1	4 ± .4	12 ± .5
Fe	Isolated lipopigment	266 ± 24	2737 ± 597	162 ± 48	432 ± 164
	Total affected	285 ± 80	1119 ± 323	91 ± 9	96 ± 9
	Total control	291 ± 10	615 ± 6	71 ± 3	70 ± 2
Ca	Isolated lipopigment	1120 ± 244	3923 ± 83	1216 ± 352	4054 ±1985
	Total affected	164 ± 10	475 ± 150	391 ± 114	983 ± 224
	Total control	72 ± 18	150 ± 10	160 ± 8	500 ± 20
Mo	Isolated lipopigment	18 ± 3	15 ± 6.8	11 ± 2.7	92 ± 29
	Total affected	2.8 ± .5	1.8± .3	.3± .06	< .2
	Total control	4.1 ± .2	1.7± .1	.3± .03	.05± .01
Sr	Isolated lipopigment	11 ± 3	33 ± .7	7 ± 1	90 ± 45
	Total affected	.9 ± .22	1 ± .30	1.2± .09	1.7 ± .6
	Total control	.2 ± .01	.7± .03	.9± .10	3.2 ± .6
Mn	Isolated lipopigment	9 ± 1	13 ± 6	7 ± 2	35 ± 18
	Total affected	7.5 ± .5	2.9± .4	6.4± .7	1.7 ± .2
	Total control	15 ± .4	6 ± .2	7 ± .3	1 ± .1

Table 10 continued

Al	Isolated lipopigment	86 ± 8	75 ± 18	103 ± 20	337 ± 117
	Total affected	32 ± 12	15 ± 7	36 ± 10	27 ± 11
	Total control	N.A. [a]	N.A.	N.A.	N.A.
Ni	Isolated lipopigment	55 ± 28	<10	17 ± 4.1	49 ± 8.3
	Total affected	N.D. [b]	N.D.	N.D.	N.D.
	Total control	N.A.	N.A.	N.A.	N.A.
Cr	Isolated lipopigment	<10	<10	15 ± 3.9	83 ± 38
	Total affected	N.D.	N.D.	N.D.	N.D.
	Total control	N.D.	N.D.	N.D.	N.D.
Zn	Isolated lipopigment	279 ± 54	136 ± 29	152 ± 32	242 ± 76
	Total affected	129 ± 11	115 ± 8	88 ± 30	92 ± 10
	Total control	133 ± 6	107 ± 3	62 ± 2	65 ± 1
Mg	Isolated lipopigment	1471 ± 431	1213 ± 221	1389 ± 723	1011 ± 739
	Total affected	516 ± 16	721 ± 51	699 ± 124	600 ± 74
	Total control	930 ± 20	1010 ± 16	1080 ± 60	850 ± 20
Na	Isolated lipopigment	828 ± 258	3456 ± 1690	11963 ±8809	2198 ± 445
	Total affected	5430 ± 1330	9803 ± 1852	3533 ± 544	7753 ±1070
	Total control	2150 ± 40	7640 ± 240	3600 ± 160	5500 ± 650
K	Isolated lipopigment	<200	<1000	1823 ±1353	636 ± 218
	Total affected	6110 ± 388	6403 ± 857	9230 ±1815	6855 ± 371
	Total control	11100 ± 150	12900 ± 190	14400 ± 400	16000 ± 200

a N.A. Not assayed for
b N.D. Not present in detectable amounts.

Concentrations of individual metals in the lipopigment varied considerably, depending on the tissue of origin. The highest concentration of any particular metal was copper in the liver lipopigment bodies, but the copper content of lipopigment isolated from the other tissues was much lower. When calculated as an enrichment of the total control tissue concentration, the liver lipopigment copper content was not exceptional. Its accumulation in the lipopigment bodies was 27 times the total liver value, whereas the relative enrichment of copper in brain lipopigment was 38 times. There was significantly more iron in kidney lipopigment than in the lipopigment from other tissues, but the specific enrichment over the total tissue concentration was only 4.5 times. Calcium was enriched in lipopigment from all tissues compared to total control tissue concentration being 26 times for kidney derived lipopigment. Zinc was also enriched in all the lipopigments.

The concentration of copper in total affected liver was twice that of the control tissue, as were the affected liver, kidney, pancreas and brain calcium concentrations. Total affected and control tissue concentrations of the other metals were similar to each other.

Molybdenum, strontium, manganese, aluminium, nickel and chromium were present in lipopigment in all tissues, at concentrations generally lower than those of the metals listed above. Their enrichments over the total tissue values were generally greater in the brain lipopigment where these metals specifically accumulate, most notably molybdenum which is enriched 1800 times.

(b) Metals in CSF: The concentration of a number of metals in the CSF of 3 sheep affected with ceroid-lipofuscinosis was measured and compared with values obtained in CSF from four normal and five heterozygous animals. No differences were observed between the normal and heterozygote animals, which were considered together as controls (Table 11). For all metals, the range of control values obtained spanned the individual affected sheep CSF concentrations. No metals were present in the CSF of affected sheep in elevated concentrations. The major cation in CSF was sodium, 2,600 parts per million. Concentrations of potassium, calcium and magnesium were

Table 11

Metal concentrations in CSF in µg/ml

	Cu	Fe	Ca	Al	Zn	Mg	Na	K
Affected sheep 64/85	.07	1.62	43.1	.41	.60	19.3	3402	90
Affected sheep 46/85	.03	.53	30.6	.30	.22	8.7	1200	37
Affected sheep 9/85	.03	.42	50.7	.29	.05	16.9	3190	78
Mean of 9 control (5 normal and 4 heterozygous) sheep ± s.e.m	.04 ±.006	.44 ±.15	36.0 ±2.4	.46 ± .09	.35 ±.17	15.7 ± 1.5	2605 ±297	72 ±7.7

lower. Concentrations of iron, copper, aluminium and zinc were much lower being less than one part per million.

Discussion

The wide ranges in the concentrations of copper, iron, and other metals in lipopigment isolated from various tissues of sheep affected with ceroid-lipofuscinosis (Table 10) do not support any common role for these metals in lipopigment formation. Lipopigment occurs in all tissues despite their widely differing copper and iron contents. Apparent elevations in the concentration of copper in total affected liver, iron in total affected kidney, and calcium in all the affected tissues equate with the high concentrations of these metals in the lipopigment in these tissues, and normal concentrations in the rest of the tissue.

The concentrations and distributions of these and the other metals studied in the lipopigments from different tissues can be explained in terms of the lipopigment cytosomes functioning as lysosomes, at least in so far as metal metabolism is concerned. The specific role of various organs in the metabolism of particular metals must also be taken into account. The tissue specificity of lipopigment copper accumulation, 1.3% of liver derived lipopigment, can be explained in this way. Total tissue concentrations show that this specificity is not a primary property of the lipopigment but is proportional to the widely differing overall copper concentrations in each tissue. (Table 10). Copper is preferentially stored in liver in normal animals and it is this organ that regulates copper balance (Sternlieb, 1980). Excess copper is stored in lysosomes (Lindquist, 1968; Gooneratne et al., 1979; Johnson et al, 1981; Helman et al., 1983 and Helman et al., 1985), possibly in a distinct heavy lysosome population (Porter, 1974). Overload of this system in sheep results in centrilobular necrosis, release of copper and a haemolytic crisis (Gooneratne et al., 1979; Kelly, 1985). This has not been observed in any of the sheep affected with ceroid-lipofuscinosis. As two thirds of the lipopigment is protein, the copper content of the liver lipopigment is similar to the liver nuclear fraction copper content in prehaemolytic copper dosed sheep (Gooneratne et al., 1979). The high copper content in the liver derived lipopigment is therefore not excessive

for a lysosome derived organelle in a healthy sheep.

Lysosomes have been shown to be centrally involved in the metabolism of a number of other transition and heavy metals, such as aluminium (Gruca and Wisniewski, 1984), plutonium (Seidel et al., 1985), gold, mercury, lead and tellurium (Sternlieb and Goldfischer, 1976). Sequentration into lysosomes is a method of regulation of cellular homeostasis of a number of metals, e.g. calcium concentrations in neutrophil cytosol (Klempner, 1985). For some elements it may be the only mechanism to avoid toxic concentrations occurring in the cytoplasm. At moderate loadings of a number of metals, lysosome derived lipofuscin-like bodies appear which contain the sequestered metal (Sternlieb and Goldfischer, 1976).

In post-mitotic cells this storage of metals is likely to be cumulative. This argument has been used to explain the age related increase in copper, iron and zinc concentrations in neuronal lysosomes in the rat (Brun and Brunk, 1973). The specific accumulation of molybdenum, strontium, manganese, aluminium, nickel and chromium in the brain derived lipopigments (Table 10) probably comes about in a similar way. These metals are sequestered into the lysosome derived lipopigment in all tissues. In visceral tissues the lipopigment is lost upon death of cells containing it but post-mitotic neurones are long-lived cells and so storage is cumulative, i.e. lipopigment in them contains the additive history of metal sequestration in the animal.

This study also shows the value of an extensive survey of metal accumulation in a variety of tissues before conclusions on pathogenic mechanisms are drawn from data. Had only the liver lipopigment metal concentrations been determined, a role for copper in the pathogenesis of ovine ceroid-lipofuscinosis could have been reasonably, but erroneously, advanced. Particular accumulations of other metals in lysosome derived organelles in certain tissues may arise in the course of normal metabolism of the metal. For example there is confusion as to the role of aluminium in Alzheimer's disease (McDermott et al., 1979; Galle et al., 1980; Crapper et al., 1980). More knowledge on the excretion, storage and mechanisms used to maintain homeostasis of this metal, and the organs involved, could contribute to the debate.

The concentrations of all the metals measured in CSF from affected sheep did not significantly differ from the control values (Table 11). In particular the concentration of iron was not elevated in the CSF of affected sheep. It was close to the reported human control CSF concentration of 0.45 µg/ml whereas iron concentrations reported for CSF from patients with infantile and juvenile ceroid-lipofuscinosis were respectively 8 and 9 times this value (Johannson et al., 1984). It is difficult to avoid some erythrocyte contamination when obtaining CSF. As CSF is a hypotonic solution these erythrocytes may lyse. Adventitious iron released from haemolysed erythrocytes can therefore contribute to apparent CSF iron concentrations. In the present experiments erythrocytes were found even in apparently colourless CSF and the CSF iron concentrations (Table 11) should therefore be regarded as maximal rather than actual values. Freezing of CSF would exacerbate this problem.
A similar source of error may therefore contribute to the apparent differences in the CSF iron contents reported between patients with ceroid-lipofuscinosis and controls (Gutteridge et al., 1982a; Johansson et al., 1984), especially as the CSF samples were taken in different places at different times.

The data presented here do not support a defect in iron metabolism being involved in the pathogenesis of the ceroid-lipofuscinoses, nor do they provide evidence for a role of iron or copper catalysed peroxidation in lipopigment formation. This is in accord with the conclusions reached in chapters III, IV and V based on lipid and fatty acid analyses. As desferrioxamine treatment can have serious harmful side-effects (Halliwell et al., 1985; Halliwell and Gutteridge, 1986) its use as an iron chelator in the treatment of ceroid-lipofuscinosis (Westermarck and Santavuori, 1984) may not be in the patients' interest and should be reconsidered.

CHAPTER VII

GENERAL DISCUSSION

Systematic biochemical analyses of lipopigment cytosomes isolated from sheep affected with ceroid-lipofuscinosis reveal a very different composition to that inferred from histochemical and ultrastructural studies. Rather than "lipoid" the lipopigment is 70% protein (Table 8, Chapter V). Most of the remainder is lipid. There is no indication of any involvement of lipid peroxidation in lipopigment formation. The density of the lipopigment is consistent with its composition reported here, rather than with a mainly lipid structure that would have a density close to, or less than, one.

The structural appearance of membranous arrays, and the apparent similarity of the lipopigment to similarly stained electron micrographs of multilamellar liposomes (Stoeckenius, 1962; Finer et al., 1972) is also deceptive. In fact these two structures have little in common. Multilamellar liposomes are very susceptible to osmotic shock and sonication (Johnson et al., 1971; Finer et al., 1972) whereas the lipopigment was shown in these studies to be refractory to these processes. This resistance to sonication and osmotic shock allows the lipopigment to be isolated by the methods used and distinguishes it from any membranous structure made up of "Singer and Nicolson" (1972) type bilayers. The high proportion of protein is also difficult to accommodate in a bilayer structure. Amongst mammalian membranes only the inner mitochondrial membrane has a similar protein to lipid ratio (De Pierre and Ernster, 1977). In this case, many of the proteins are not intrinsic membrane proteins but are large molecules mainly in the aqueous phase anchored to the membrane by a short hydrophobic sequence. They stand out from the membrane surface in electron micrographs. No such proteins have been observed in the lipopigment in ovine ceroid-lipofuscinosis (R.D.Jolly, pers. comm.), nor reported in the lipopigment in the human forms of the disease.

The lipopigment lipids are not consistent with a bilayer structure either. The proportion of phospholipids is low, i.e. 10-15%,

and one of the major ones, $(MAG)_2P$, is a different shape to normal bilayer phospholipids. It has only one fatty acid chain per glyceryl unit, rather than the normal space filling two. Free fatty acids prefer a micellular arrangement. Ubiquinone does not fit into the phospholipid palisade of bilayer membranes but probably sits parallel to the plane of the membrane in the hydrophobic core between the two sets of acyl chains (Alonso et al., 1981; Katsikas and Quinn, 1983; Aranda and Gomez-Fernandez, 1985; Ulrich et al., 1985; Ondarroa and Quinn, 1986). Both dolichol and dolichyl esters have been shown to be disruptive of bilayer membranes (Valtersson et al., 1985), which is not surprising considering their structures. Dolichol with 90-105 carbons is far too long just to transverse a bilayer membrane, a distance of only 40-50 carbons. For dolichyl esters the problem is greater. Of the lipopigment neutral lipids only cholesterol can be accommodated in a bilayer palisade.

Whatever the structural arrangement of the components of the lipopigment, it must accommodate the following features:

(i) It must be compatible with the stoichiometry of the lipopigment components.
(ii) Its refractory nature to osmotic shock and sonication indicate that it must be "solid" rather than "fluid".
(iii) Its lipid staining characteristics must be explainable.
(iv) The components of the lipopigment must be arranged in a way that gives rise to a multilamellar appearance.
(v) The fluorescence of the lipopigment, a property that has bedevilled so much research into the ceroid-lipofuscinoses, must be explainable.

Further experimental data are needed for a firm hypothesis to be advanced on the structural nature of the lipopigment. However all the above points can be explained in terms of a "Danielli and Davson" (1935) type structure, with alternate layers of lipid sandwiched between layers of hydrophobic protein. Histological lipid stains stain for hydrophobic areas, rather than for specific functional groups. In order for the lipopigment to appear "lipoid", it must have large hydrophobic areas. These could be the lipids or equally hydrophobic protein.

The fluorescence of the lipopigment in the ceroid-lipofuscinoses has previously led to the assumption that it contains a large amount of a strong fluorophor, fluorescent under any conditions, but this need not be the case. Fluorescence of a compound can be highly dependent on its environment, a property of some molecules that has proved useful in determining structural arrangements in membranes (Radda, 1975; Shinitzky and Barenholz, 1978); for instance the orientation of ubiquinones of varying chain length (Katsikas and Quinn, 1983). Fluorescent spectra are not "characteristic" in the sense that the lipopigment fluorescence is claimed to be. Absorptions and emissions can occur at similar wavelengths in quite different compounds, for the same reasons as in UV-visible spectra. The observed fluorescence of anhydrous protein suspended in glycerol (Chapter IV) can alone account for the fluorescence of the lipopigment, if the lipopigment protein provides its own hydrophobic environment. Other molecules, that have fluorescent properties highly dependent on their environment may also contribute, without there being any "characteristic fluorophor" at all. It is also worth noting that lipids quickly oxidise to fluorophors when exposed to air, and such artefacts are almost bound to be generated in prolonged lipopigment isolation and lipid extraction procedures as used by previous workers.

There is considerable evidence for such a "Danielli and Davson" (1935) type of assembly in "tubular myelin like" multilamellated structures that occur in the lungs of patients with pulmonary alveolar proteinosis (Hook et al., 1978; 1986). This disease has long been recognised as a proteinosis (Rosen et al., 1958). Antibody labelling has been used to demonstrate the abnormal protein content (Singh et al., 1983). The abnormally occurring bodies in this disease have some structural similarities to the lipopigment in ceroid-lipofuscinosis.

Because of the confusion surrounding the pathogenesis of the ceroid-lipofuscinoses they are often not considered amongst the lysosomal storage diseases, e.g. (Stanbury et al., 1972; Glew et al., 1985). On the evidence presented here they should be. None of the current studies contradict the long-held histological concept of a lysosomal involvement in lipopigment formation.

Added to that is the lysosomal nature of the lipopigment lipids (Chapters IV and V). Furthermore, analysis of the lipopigment metal content suggests that at least at some stage in its biogenesis the lipopigment is part of a functional lysosomal structure, able to carry out normal lysosomal metal metabolism (Chapter VI). The specific accumulation of some non-excreted metals in brain lipopigment suggests that this capacity remains for an extended period. This may seem surprising in view of the isolated lipopigment structure, but it should be noted that any limiting membrane surrounding the lipopigment in vivo would be destroyed by the isolation procedure, with the concomitant loss of soluble lysosomal components. Low molecular weight proteins are the major species present in the lipopigment. Like the storage material in other lysosomal storage diseases, they are abnormally stored to the extent that their presence allows the distinction to be made between total control and affected tissue homogenates (Chapter V). On these grounds ovine ceroid-lipofuscinosis qualifies as a lysosomal proteinosis. No inherited lysosomal proteinoses have been recognised as such before, but there are a number of possible mechanisms that would lead to the abnormal accumulation of proteins in lysosomes.

Most cellular proteolysis is lysosomal (Hershko and Clechanover, 1982; Mayer and Doherty, 1986) and two classes of lysosomes are involved, one being of similar density to the lipopigment cytosomes (Neely et al., 1977; Mortimer and Surmacz, 1984). The rate of lysosomal proteolysis is responsive to serum concentrations of amino acids, especially those suitable as substrates for gluconeogenesis (Mortimore and Poso, 1984), and possibly to other amino acid derived metabolites (Grinde, 1984). It is also responsive to glucagon and insulin levels (Schworer and Mortimore; 1979; Aronson, 1980). These findings suggest that it is a process of metabolic as well as of catabolic importance.

In order for lysosomal proteolysis to function in this manner it must be highly regulated, to allow the independent regulation of the degradation of specific proteins and the quantity of each degraded (Huston and Mortimore, 1982). Cellular function would be seriously impaired if for example the long-lived proteins that determine phenotypic expression were not protected against lysosomal proteolysis.

On the other hand there is a requirement for the rapid catabolism of fast acting protein hormones. Both proteins would lose their efficacy if they were not segregated from randomly mixed proteins and protein fragments that are catabolised to amino acids to fulfill metabolic demand.

Sequestration of proteins into lysosomes or prelysosomal subcellular organelles is normally the committed and rate limiting step in protein degradation (Dean, 1975a; 1975b; Ward et al., 1977; Schworer et al., 1981). Some control is evident at this level (Seglen et al., 1980), and different proteins enter the lysosomal compartment by different mechanisms (Grinde, 1985; Seglen et al., 1985; Glaumann et al., 1985; Khairallah, 1985) allowing further discrimination. However because entry into the lysosomal system is rate limiting, little is known of subsequent steps. Intermediates in the degradation pathway are rapidly catabolised with the concomitant loss of such experimental determinants as antigenicity (Beynon et al., 1985) which makes tracing the fate of any particular protein difficult. One approach to this problem is the use of lysosomal protease inhibitors, that slow lysosomal proteolysis and make it the rate limiting step (Seglen et al., 1985). For instance leupeptin, a lysosomal protease inhibitor causes the accumulation of dense and complex vesicles in hepatocytes after in vivo administration (Kovacs et al., 1985). Dense bodies have also been observed in neurones of rat brains into which leupeptin had been perfused. They formed quickly, their formation was reversible, and they had structural and fluorescent properties that drew attention to their similarity to lipofuscin and lipopigments in the ceroid-lipofuscinoses (Ivy et al., 1984). It may be of interest that leupeptin has also been shown to block certain learning tasks in rats (Staubli, 1985).

A number of lysosomal proteases have been described, some with quite particular specificities (Barrett and Heath, 1977). Other lysosomal proteases have subsequently been described (e.g. Tanaka et al., 1984; Liao and Lenney, 1984; Muto and Tani, 1985) and there is no reason to believe that all the enzymes involved have been discovered. There are also endogenous inhibitors of lysosomal proteases (e.g. Katunuma and Kominami, 1983).

The role of proteases in this process does not stop with intralysosomal proteolysis. Proteases are also involved in the control mechanisms. Limited proteolysis may signal the initial commitment of proteins to intralysosomal degradation (Grinde, 1985). Different levels of cathepsins in different tissues suggest that they may be involved in specialised cellular functions other than bulk intralysosomal protein degradation (Segundo et al., 1986). Specific lysosome associated proteases may also be required for the activation of certain lysosomal enzymes from "zymogen like" precursors. This is so in yeasts (Ammerer et al., 1986; Woolford et al., 1986). As in these organisms current knowledge of proteases "is likely to be only the tip of the iceberg" (Achstetter et al., 1984).

Inheritance of ovine ceroid-lipofuscinosis is as an autosomal recessive trait, suggesting that an enzyme defect is involved. If the accumulated protein was a direct consequence of abnormal protein synthesis a dominant inheritance would be expected. The simplest rationale for the accumulation of low molecular weight proteins in lysosome derived organelles observed in this study is that the defect is of a lysosomal protease. Given the control requirements for lysosomal proteolysis, a defect in the control of this process is also possible. Perhaps only the kinetics of lysosomal proteolysis are affected, and the stored polypeptide fragments are degraded, albeit at a slower rate than normal, resulting in a larger than normal intralysosomal steady state pool of proteolysis substrates.

No inborn errors of lysosomal protein catabolism have been recognised as yet but there is no a priori reason why they should not occur. In fact the possibility of such diseases has previously been advanced (Dean, 1975a). Their lack of occurrence was explained by a postulated incompatibility with life. However that was before the nature of the lipopigment in ovine ceroid-lipofuscinosis was recognised, and before the complexity and consequent subtlety of lysosomal proteolysis became apparent. It is also possible that there exists somewhere in the cell a normal mechanism to degrade the stored polypeptides or their precursors and that the inborn error causes their erroneous presentation to the lysosomal system. Abnormal post-translational modification of protein may be involved. A failure in non-lysosomal proteolysis could also result in the

accumulation of non-degradable fragments in lysosomes.

This list of possible pathogenic mechanisms is not exhaustive. Further experimentation is required before any firm conclusions can be reached as to which mechanism is operating in ovine ceroid-lipofuscinosis, or the other ceroid-lipofuscinoses. Since ovine ceroid-lipofuscinosis is a good model for the other ceroid-lipofuscinoses they are also likely to arise in a similar way. Different mutations affecting the same protein, or mutations affecting different closely related proteins could account for the previously unexplained subtypes of the ceroid-lipofuscinoses.

Whatever the underlying defect in ovine ceroid-lipofuscinosis several criteria must be met for the disease to be compatible with life. The abnormally stored proteins must have something in common that causes their degradation to be impaired. If a large proportion of proteolysis substrates were so affected, viability of even a foetus would seem unlikely. This suggests that the proteins whose degradations are disturbed are limited. Even though the extent of heterogeneity of the stored proteins is unclear, the finding of stored polypeptides of specifically defined molecular weights (Chapter V) supports this view. Such circumstances would arise if the catabolism of only some rare protein(s) were affected. Alternatively a highly specific protease responsible for the cleavage of an uncommon amino acid sequence could be missing. Given the degeneracy of lysosomal proteolysis, with both C and N terminal peptidases as well as endopeptidases being involved (Barrett and Heath, 1977), it is possible that some other concomitant event is required. For instance the N terminal blockage of a small proportion of proteins by glycation may block degradation from that end. The absence of a C terminal peptidase may then become apparent, whereas for normal proteins its lack of activity could be covered for by degradation from the N terminal end.

The studies presented here have implications for other fields of research besides the ceroid-lipofuscinoses. Whatever the nature of the primary lesion(s) in these diseases their elucidation may give an insight into the normal mechanisms of lysosomal proteolysis. As stated earlier, because initial commitment to this process is normally

rate limiting, subsequent steps are hard to study. Ultrastructural studies in ceroid-lipofuscinosis, where the rate limiting step may be changed, could provide some indication of subsequent steps in this process, for example on the debated role of transport vesicles. It is of note that normally unseen "presumptive transport vesicles" have been revealed in studies of I-cell disease affected fibroblasts (Van Dongen et al., 1985).

Cytosomes with similar morphological and staining properties to the lipopigment in ceroid-lipofuscinosis have been observed after treatments with a number of drugs that perturb lysosomal function (Lullman et al., 1978; Rees, 1978; Buchmeim, 1979; Blok et al., 1981; Lullmann and Lullmann-Rauch, 1981; Furuno et al., 1982; Christensen et al., 1983; Jagel and Lullmann-Rauch, 1984; Glaumann et al., 1986). As mentioned earlier the effect of leupeptin has been recognised as causing a proteinosis (Furuno et al., 1982; Ivy et al., 1984). In other cases an induced lipidosis has been invoked to explain similar observations (Lullmann et al., 1978; Buchheim et al., 1979; Lullmann and Lullmann-Rauch, 1981; Christensen et al., 1983; Jagel and Lullmann-Rauch, 1984). The bases of such descriptions are largely the same as those previously used for the ceroid-lipofuscinoses; the apparent lipoid nature of the abnormally occurring cytosomes as indicated by ultrastructural and histochemical studies. The same weaknesses apply. Some of these induced diseases may therefore also be proteinoses. There is evidence for the accumulation of protein in chloroquine and chlorphentermine induced "lipidoses" (Blok et al., 1981; Christensen et al., 1983). Some of the drugs that induce so-called "lipidoses" may be specific inhibitors of lysosomal proteolysis, but this need not be a precondition for a proteinosis. Even drugs that cause a generalised disturbance in lysosomal function, for instance by increasing the intralysosomal pH, are likely to affect lysosomal proteolysis more than other lysosomal catabolic pathways if it is indeed the most sensitive and regulated of lysosomal degradative pathways.

The "dense bodies" observed in some of these diseases and in metabolic perturbations of lysosomal proteolysis are likely to have a high protein to lipid ratio. Their apparent multilamellar membranous appearance is therefore likely to be deceptive for the same reasons as

for the lipopigment in ceroid-lipofuscinosis. The same constraints on a "Singer and Nicholson" (1972) type bilayer membrane structure are likely to apply.

Many of the properties of the lipopigment from tissues of sheep with ceroid-lipofuscinosis have also been reported for the age pigment lipofuscin. There is a similar heterogeneity of fluorophors in lipofuscin from human heart (Siebert et al., 1962; Hendley et al., 1963a,b) and retinal pigment epithelium (Eldred et al., 1982). Non-TLC-migrating fluorescent lipoid material is only a minor component in lipofuscin from human liver which contains the normal tissue phospholipids. $(MAG)_2P$ is a component of lipofuscin from a variety of tissues (Siakotos and Koppang, 1973). Similarly, dolichol is a significant component of brain lipofuscin (Pullarkat and Reha, 1982) and is enriched in most tissues where lipofuscin accumulates (Pullarkat et al., 1984). The dolichol content of many tissues increases with age (Keller and Nellis, 1986). Protein is the major component of heart lipofuscin and preliminary experiments suggest that lipofuscin has a high protein to lipid ratio (Siebert et al., 1962; Hendley et al., 1963b; R.D. Jolly pers. comm.). The components in its multilamellar structures may be similarly organised to those in the ceroid-lipofuscinosis lipopigment rather than multiple layers of Singer and Nicholson (1972) type biomembranes. Ceroid-lipofuscinosis has been presented as a model for aging (Armstrong and Koppang, 1981). The similarities between lipofuscin and the lipopigment in ceroid-lipofuscinosis suggest that this is valid, albeit for different reasons to those usually advanced. Other lipofuscin-like lipopigments may also be largely proteinaceous, and the consequence of general disruptions in lysosomal function particularly affecting lysosomal proteolysis. More experimental data are required to unravel the inter-relationships between these lipopigments. In the interim a peroxidative origin for any of them should not be regarded as axiomatic.

Recognition of ovine ceroid-lipofuscinosis as a lysosomal proteinosis is a considerable advance in the understanding of these diseases. Because it is an accurate model of the human disease it may have consequences of a directly medical nature. The failure to find any justification for desferrioxamine therapy (Chapter VI) suggests that

this treatment should be re-evaluated. Sensitive methods for the detection of the abnormally stored polypeptides could open a way for prenatal diagnosis of the disease. Suitably modified isolated stored polypeptides may be useful as substrates for the affected enzyme activity, and could be useful in heterozygote detection. The achievement of any of these ends would help alleviate the human suffering caused by the ceroid-lipofuscinoses, even before their pathogenesis is fully understood.

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PUBLICATIONS

Much of the work presented in this thesis has already been published in the following articles :

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.

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A normal-phase HPLC technique for the quantification of ubiquinone, dolichol and cholesterol developed during this work has also been published :-

Palmer, D.N., Anderson, M.A. and Jolly, R.D. (1984). Separation of some neutral lipids by normal-phase high-performance liquid chromatography on a cyanopropyl column : Ubiquinone, dolichol and cholesterol levels in sheep liver. Anal. Biochem., **140** 315-319.