BIOGENESIS AND SIGNIFICANCE OF LIPOFUSCIN IN THE EQUINE THYROID GLAND.

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ABSTRACT

Yellow-brown granules of lipofuscin (age pigment) accumulate in the cytoplasm of long-lived cells of many eucaryotes, including man. The granules are derived from lysosomes, and are defined by characteristic morphology, colour, fluorescence, and histochemistry. They increase in number with age in post-mitotic cells, and are regarded as markers of the aging process. Lipofuscin is widely assumed to consist of products of peroxidised fatty acids cross-linked with amino groups of proteins, nucleic acids, and phospholipids, but this theory has not been proven.

The occurrence, histology, structure, and composition of lipofuscin in thyrocytes from horses of a wide range of ages were studied. Granules were absent at birth but were widespread by the age of 5 years. In young horses, granules were largest in follicles which contained abnormal colloid. After 5 years the amount of lipofuscin in thyrocytes was not age-related. Lipofuscin was fluorescent, and stained with PAS, Schmorl's, and Masson's Fontana stains, although the staining intensity varied between horses. Lectin histochemistry demonstrated the presence of mannose and/or glucose. Small subpopulations of granules in some sections contained iron and some granules contained DNA.

Lipofuscin granules were irregular in size and shape, and consisted of an electron-dense matrix and relatively electron-lucent 'vacuoles', which did not contain lipid. They appeared to fuse with colloid droplets, and small granules were observed within colloid droplets.

Thyroid lipofuscin granules isolated by osmotic shock and differential centrifugation showed the same ultrastructure as *in situ*. The protein content ranged from 15% to 77% w/w (mean = 36%). In contrast, the amino acid composition was constant and similar to that of thyroglobulin. The most distinctive feature of lipofuscin protein was the presence of four proteins of 14-18 kDa. The halide

concentration of lipofuscin was approximately twice that of thyroid tissue. Lipofuscin contained no triglycerides, and only small quantities of phospholipids (mean = 1.25% w/w). In contrast, the concentrations of cholesterol and dolichol, up to 19% and 15% respectively, were high although very variable. Small amounts of nuclear DNA were detected. The empirical formula of the rest of lipofuscin indicated that it was principally carbohydrate, which was consistent with the PAS and lectin histochemistry.

Most of the lipofuscin mass was analysed and there was no evidence that it arose from lipid peroxidation. The protein fraction of thyroid lipofuscin probably consists of proteolytic intermediates of thyroglobulin. Components from other sources, and turnover of granule contents, contribute to the chemical heterogeneity.

The composition of lipofuscin probably varies between tissues. However all lipofuscins are likely to share lysosomal functions such as storage of proteolytic intermediates, metals and dolichol. As with thyroid lipofuscin, they may be more soluble and more readily analysed than is generally assumed in the literature. They may also be active organelles of metabolic significance, rather than inert indicators of the aging process. On the basis of this study, they should be re-evaluated.

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ABBREVIATIONS

AFIP	Armed Forces Institute of Pathology
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
DIT	diiodotyrosine
EDTA	ethylenediaminetetraacetic acid
Feulgen-NAH	Feulgen-Naphthoic acid Hydrazine
HPLC	high performance liquid chromatography
H&E	haematoxylin and eosin
IgG	immunoglobulin G
kDa	kiloDalton
LDS	Lithium dodecyl sulphate
LDS-PAGE	LDS-polyacrylamide gel electrophoresis
MIT	monoiodotyrosine
NMR	nuclear magnetic resonance
PAS	periodic acid-Schiff
PBST	phosphate-buffered saline/Tween 20
PVDF	Polyvinylidene difluoride (Immobilon™, Millipore Corporation, MA.)
TBS	Tris-buffered saline
TLC	thin layer chromatography
TRH	TSH-releasing hormone
Tris	Tris(hydroxymethyl)aminomethane
Trizma™	Tris(hydroxymethyl)aminomethane (Sigma)
TSH	Thyroid stimulating hormone
TTBS	Tween 20/Tris + Cations
T3	Triiodothyronine
T4	Tetraiodothyronine, = thyroxine

CHAPTER 1

INTRODUCTION

The lipopigments are a heterogeneous group of intracytoplasmic granules which share characteristics of colour, morphology, fluorescence, and affinity for a number of histological stains. The literature regarding the lipopigments is vast, contradictory, and plagued by confusion.

Numerous terms are used to describe and distinguish lipopigments, including lipofuscin, ceroid, age pigment, wear-and-tear pigment, cytolipochrome, chromolipoid, and haemoceroid. This variety of terms reflects not only the heterogeneous nature of lipopigments, but also the considerable confusion which exists in the literature concerning their origins and relationships. One simplified system of terminology has been to refer to lipopigments which appear as a result of a pathological process as 'ceroid' and those that appear spontaneously and accumulate progressively with age in normal tissues as 'age pigment' (Oliver 1981, Porta 1987). The term 'lipofuscin' [Greek:lipo-=fat + Latin:fuscus=dusky] was introduced in 1922 by Borst to describe the yellow pigment observed in neurones by Hannover in 1842 and correlated with age by Koneff in 1886 (Porta and Hartroft 1969). 'Lipofuscin' is used as a synonym for age pigment by most authors (Oliver 1981, Porta 1987) but has also sometimes been used to describe induced or pathological lipopigments by others. An assumption that the lipopigments have an origin and composition in common, and that therefore the terms 'lipofuscin' and 'ceroid' are synonymous (e.g. Bieri *et al* 1980) exacerbates the confusion.

The occurrence of ceroid in association with Vitamin E deficiency is well recognized (Lillie *et al* 1941 1974, Moore and Wang 1947, Sulkin and Srevanij 1960, Cordes and Mosher 1966, Bieri *et al* 1980, Katz *et al* 1985, Sarter and van der Linde 1987, Davies *et al* 1987, Porta 1987). Since Vitamin E is believed to act as an antioxidant *in vivo*, the conclusion that ceroid is an endproduct of peroxidation of lipid is almost universally accepted, although not proven chemically. Extrapolation of this theory has led to the widely held belief that

oxidative damage to cellular lipids and proteins, initiated by free radicals, is also the cause of age pigment formation and accounts for its fluorescence under ultraviolet light (Chio and Tappel 1969a,b, Davies 1988). Since age pigment accumulates in an age-dependent fashion, such peroxidation has also been suggested as being implicated in the aging process. An entire theory that aging is due to free radical-induced oxidative damage has been developed (Harman 1984, 1988), for which the accumulation of age pigment is considered supporting evidence (Lippman 1983, Gordon 1974). Thus a curious paradox exists in that although age pigment is generally regarded as a 'normal' cellular inclusion related only to aging, and ceroid as being a consequence of pathological change, the presumed biogenesis of the 'abnormal' lipopigment has been extensively used as a valid model for the composition, biogenesis, and significance of the 'normal' lipopigment. The distinction between the two lipopigments, and the differences between them, are frequently understated, leading to such unjustified conclusions (Porta 1987).

For the purposes of this thesis, the term 'age pigment' is used to describe the granules which accumulate progressively with age in normal tissues, and the term 'lipofuscin' is used as a more general term for granules which accumulate in normal tissues but have not been demonstrated to increase with age, unless the author/s cited have used the term age pigment. 'Ceroid' is used to describe granules which develop in association with pathological processes, particularly where there is reason to suppose that lipid peroxidation may be involved. The term 'lipopigment' is used as a general term encompassing age pigment, ceroid, and lipofuscin.

Characteristics of lipofuscin

Colour

Lipofuscin has a natural gold-yellow or brown colour which may cause a gross discolouration of tissues (Dolman and Macleod 1981) but the chemical nature of the chromogenic group is unknown (Elleder 1981). There is evidence that lipofuscin has little or no colour when newly formed, and develops colour with time (Few and Getty 1967, Pearse 1985).

Staining

Lipofuscin remains intact in paraffin sections but stains with lipid stains such as Sudan Black B and Oil Red O (Dolman and Macleod 1981), although the intensity of sudanophilia varies considerably (Elleder 1981). Lipofuscin reduces ferric ferricyanide to Turnbull's blue (Schmorl's stain, Pearse 1985) but the identities of the reducing groups are unknown (Elleder 1981). A positive reaction to periodic acid-Schiff (PAS) stain is variously attributed to the presence of ethylenic groups or to the presence of a glycoprotein (Dolman and Macleod 1981). The reaction of periodic acid with hexoses produces aldehydes which then react with Schiff's reagent to produce Schiff bases. Theoretically aldehydes which were already present would also react with Schiff's reagent (Pearse 1985).

Lipofuscin is acid-fast after prolonged Zeihl-Neelsen staining. This property has been attributed to the possible presence of acid groups (Dolman and Macleod 1981) or to polymerization of lipids giving rise to structures resembling mycolic acids of tubercle bacilli (Elleder 1981). RNA and a number of amino acids have been demonstrated histochemically in lipofuscins (Dolman and Macleod 1981). Histochemical reactions for a number of enzymes, particularly the lysosomal enzymes acid phosphatase, beta-glucuronidase, and esterase, and the mitochondrial enzymes succinic dehydrogenase and cytochrome oxidase, have also been reported (Dolman and Macleod 1981).

Fluorescence

Lipofuscin exhibits fluorescence under ultraviolet light, with the reported colour of emitted light ranging through yellowish-white, yellow, orange, red-brown, to brown (Elleder 1981). Much has been inferred from this property, which has been regarded as evidence that lipofuscin is composed of the products of reactions between the amino groups of proteins and/or nucleic acids and malondialdehyde (= malonaldehyde), a product of oxidation of polyunsaturated fatty acids (Chio and Tappel 1969a,b, Kikugawa 1988). Products of an <u>in vitro</u> model of this reaction share with lipofuscin a yellow colour and fluorescence under ultraviolet light. These properties are attributed to the formation of conjugated Schiff bases. (Chio and Tappel 1969a,b). The fluorescence emission of an <u>in vitro</u> model of

conjugated Schiff bases is blue while lipofuscin granules emit yellow fluorescence (Eldred et al 1982). No fluorescent molecule extracted from any source of lipofuscin has ever been purified and chemically characterised (Eldred 1987). Although Shimasaki et al (1977) found a chloroform/methanol-soluble substance which accumulated with age in rat tissues and exhibited fluorescence similar to that reported for lipofuscin, they did not demonstrate that the substance was extracted from lipofuscin.

Morphology

Under the light microscope, lipofuscin appears as light yellow or brown intracytoplasmic granules, round or oval in shape and measuring 0.5 to 3 um in diameter. Granules are reported to increase in number, colour, and size with age (Dolman and Macleod 1981).

By electron microscopy lipofuscin granules appear as irregularly shaped, intensely osmiophilic bodies enclosed by a single unit membrane which may be so closely adherent that it is difficult to see. The granules may contain electron-lucent structures referred to as 'vacuoles' or sometimes as 'lipid droplets', which may project beyond the contour of the dense structure. These 'vacuoles' are reported to become more numerous in granules with age (Dolman and Macleod 1981). They are commonly assumed to contain lipid (Dolman and Macleod 1981, Goebel 1988) although there is a lack of evidence that the contents are lipid (Heimann 1966, Jolly in Goebel 1988). Samorajski and Ordy (1967) propose that the biogenesis of lipofuscin begins with the vacuoles, but Glees and Hasan (1976) consider that the vacuoles represent degradation of the lipopigment.

The ultrastructural appearance of the matrix of lipofuscin granules is variable; it may be homogeneous, granular, lamellated or a combination of these types (Samorajski et al 1965, Few and Getty 1967, Ikeda et al 1985). It has been suggested that the lamellated pattern is indicative of phospholipid membranes (Samorajski et al 1965).

Chemical and physical analyses

The affinity of lipofuscin for lipid stains has led to the assumption that it is principally lipid in composition. Hendley et al (1963b) reported that lipids represented only 27-40% of the mass of isolated human cardiac age pigment. In contrast, Bjorkerud (1964) found that 51% of the mass of human cardiac lipofuscin was lipid, and identified cholesterol esters, triglycerides, cholesterol, cephalins, lecithin, and sphingomyelin. Lipid polymers and phospholipids are reported to be the principal components of lipofuscin in the brain (Taubold et al 1975). However, lipofuscin is generally resistant to extraction from unfixed tissues by the chloroform-methanol methods conventionally used for lipid extraction (Elleder 1981).

Amino acids and proteins have also been found in lipofuscin (Hendley et al 1963b, Taubold et al 1975, Dolman and Macleod 1981), and protein represents 30% of the mass of human cardiac lipofuscin (Bjorkerud 1964). Approximately 30 protein bands were demonstrated by gel electrophoresis of age pigment isolated from human retinal pigment epithelial cells (Feeney-Burns et al 1988). Lipofuscin bodies of human pancreatic B-cells contain islet body peptide, as demonstrated by immunocytochemistry. The islet body peptide is located in the electron-dense parts of the lipofuscin granules, which are also acid phosphatase positive (Clark et al 1989).

The presence of carbohydrate in lipofuscin has rarely been the subject of study (Elleder 1981). However compounds labelled by concanavalin A, probably glycoproteins, are present in lipofuscin in human brain, myocardium, epididymal, and skin eccrine glands (Elleder et al 1990).

Metals, including iron, copper, calcium, and zinc have been demonstrated in lipopigments (Elleder 1981). Iron represents 0.15 - 0.2% of the dry mass of human cardiac age pigment (Hendley et al 1963b). The lipopigment which accumulates in the inherited disease ovine ceriod-lipofuscinosis contains 1-1.7% metals (Palmer et al 1988).

Lipofuscin has been found in association with melanin in some cells such as those of the retinal pigmented epithelium (Elleder 1981).

Neuromelanin, a pigment found in particular brain regions of man and some other species, is histochemically distinct from melanin found in the skin, hair, and eyes, and shares some histochemical and morphological characteristics with lipofuscin. The two pigments have been found in the same cells of the nervous system, and the similarities between them have led some authors to propose that neuromelanin contains both melanin and lipofuscin, and should be termed 'melanized lipofuscin' (Bianchi and Merighi 1986, Barden and Brizzee 1987).

Distribution of lipofuscin

Lipofuscin is typically found in neurones, which are postmitotic, and in other cells with a low mitotic rate (Porta 1987). Numerous studies, reviewed by Porta and Hartroft (1969) have demonstrated an age-related increase in age pigment in the neurones of the central nervous system in a variety of mammalian species, including man. The lipopigment granules are found in clusters in a perinuclear position (Shima and Tomonaga 1988) or at the axon hillock (Heinsen 1979). The quantity of lipofuscin accumulating varies between different sites in human (Mann et al 1978), canine and porcine brains (Whiteford and Getty 1966). Whiteford and Getty suggest that differences in lipopigment accumulation between regions in the brain may be due to differences in levels of neuronal activity, but Reichel et al (1968) report that differences in lipopigment accumulation in various parts of brains of rats and mice cannot be related to the activity level of the different areas.

Glees (1987) presented evidence, from electron microscopy of primate brains, that microglial cells can fuse with cerebral neurones, take up lipofuscin granules from them, and transport the granules to perivascular sites. Lipofuscin in astrocytes has been described by numerous authors (reviewed by Dolman and Macleod 1981).

Lipofuscin begins to accumulate in human myocardium in the second decade, after which it increases at the rate of 0.3% heart volume per decade (Ambani et al 1977). An age-related increase in lipopigment relative to myocardial volume has also been reported in canine heart (Munnell and Getty 1968).

Lipofuscin is also found in skeletal muscle (Brizzee and Ordy 1981) and there is some evidence that active muscle cells accumulate more lipopigment than relatively inactive muscle cells. Postural muscles accumulate less lipopigment than muscles of active movement, and paralysed muscles are less pigmented than muscles of active movement (Dolman and Macleod 1981).

There is a progressive increase with age in lipofuscin content of the retinal pigment epithelium. This form of age pigment appears to be derived from photoreceptor outer segments, which are constantly being shed and are phagocytosed by the cells of the retinal pigment epithelium. Phagosomes containing structures intermediate between outer segment discs and age pigment granules have been observed. Age pigment in the retinal pigment epithelium is acid phosphatase positive, and fusion of primary lysosomes with lipopigment granules has been described (Feeney-Burns et al 1988). Supporting evidence that age pigment in the retinal pigment epithelium is derived from photoreceptor outer segments comes from studies of a strain of rat, the RCS rat, which has a hereditary defect resulting in degeneration of the retinal photoreceptor cells shortly after they develop. The age-related increase in age pigment in retinal pigment epithelium cells of RCS rats is much less than that in normal rats (Katz et al 1986). Furthermore, a decrease in age-related accumulation of lipopigment in the retinal pigment epithelium of normal rats can be induced by the use of bright cyclic light, which causes destruction of photoreceptor cells (Katz and Eldred 1989). Immunocytochemistry of age pigment in retinal pigment epithelium cells has not demonstrated the presence of rhodopsin, the predominant protein of photoreceptor outer segments (Feeney-Burns et al 1988). Either rhodopsin does not enter the age pigment, or the antigenic determinants of rhodopsin are degraded or obscured within the granules so that they are not recognized by immunocytochemistry.

Lipofuscin also occurs in human oesophagus, stomach, small intestine, spleen, adrenal glands, lymph nodes, blood vessels, ovaries, uterus, (Pappenheimer and Victor 1946), pancreas (Clark et al 1989), and some cell types in the kidney (Brizzee and Ordy 1981). Pigment granules

have been observed in prostate, epididymis, Leydig and Sertoli cells of humans and animals (Brizzee and Ordy 1981, Miquel et al 1978). Lipofuscin does not begin to accumulate in seminal vesicles or epididymus until these organs become functional (Sohal 1981). Lipofuscin also occurs in murine connective tissues (Brizzee and Ordy 1981).

Studies concerning the quantity and accumulation of lipopigments must however be interpreted with caution. Since 1968, the most commonly used method of quantifying lipopigment in tissues has been by spectrofluorimetry, the measurement of fluorescent substances extracted by chloroform-methanol (Hammer and Braum 1988). This method is most unlikely to produce accurate results, because lipopigment in tissues has been shown to be resistant to extraction in chloroform-methanol (Elleder 1981). It has never been proved that fluorescent material extracted from tissues by chloroform/methanol is derived from lipopigment at all (Sohal 1987) and in fact extractable fluorescence is very poorly correlated with the number of lipopigment granules in histological section (Bieri et al 1980). A major problem is that normal tissues contain fluorescent substances that are not lipofuscin-derived. Vitamin A, its esters, and many of its derivatives and breakdown products are fluorescent under ultraviolet light (Thompson et al 1971), and this property is used to measure these compounds in tissues (Thompson et al 1971, Fung and Rahwan 1978). The fluorescence of the lipopigment which is stored in ovine ceroid-lipofuscinosis represents only a small fraction of the total fluorescence in ovine liver (Palmer et al 1986a). Furthermore important factors in spectrofluorimetry such as the temperature and the pH are very seldom specified and may not have been constant between samples in the studies undertaken (Hammer and Braum 1988), thus invalidating comparison between samples. Another common method of quantifying lipopigment in tissues is by measurement of fluorescence in histological sections (Hammer and Braum 1988). Complications of this method include calibration and fading problems (Hammer and Braum 1988).

Intracellular significance of lipofuscin

Lipofuscin granules are membrane-bound and numerous lysosomal enzymes have been demonstrated in them. They have been described as lysosomal residual bodies (Barden and Brizzee 1987, Goebel 1988). There is debate over whether the material initially forms in lysosomes or forms in some other organelle and is then stored in lysosomes. Virtually every type of organelle has been proposed for the origin of lipofuscin (Dolman and Macleod 1981).

It is widely supposed that lipofuscin is made up of indigestible, unexcreted cellular wastes (Dolman and Macleod 1981, Sohal and Wolfe 1986). Others have proposed that lipofuscin is useful, but underutilized due to the decreased capability of the cell to use it (for review see Dolman and Macleod 1981). Still others believe that lipofuscin is an active organelle (Glees and Hasan 1976). A lipopigment can be induced in neurones by hypoxia (Sulkin and Srevanij 1960) and various authors have speculated that lipofuscin may be a cellular adaptation to tissue hypoxia. It has also been proposed that lipofuscin granules represent the site of sequestration of substances noxious to the cell (Dolman and Macleod 1981).

The large amount of lipofuscin found in the cytoplasm of some cells has led to the assumption that the volume of the lipopigment must be damaging to the cells (Dolman and Macleod 1981). However, the extent to which lipofuscin affects cell function is unknown (Porta and Hartroft 1969), and there is no direct evidence that it has any deleterious effect. The function of mouse supraoptic nucleus neurones is not affected by increasing quantities of lipofuscin (Davies et al 1987).

The gradual accumulation of age pigment in postmitotic cells suggests that it cannot be excreted (Dolman and Macleod 1981). However there is evidence from ultrastructural studies of primate brains that age pigment can be transferred from neurones to glial cells by partial fusion of cell walls (Brizzee et al 1974, Glees 1987).

Other lipopigments

Lipopigments also accumulate in cells in a number of experimental and pathological conditions which have frequently been regarded as models for studying the biogenesis of lipofuscin. The term 'ceroid' was first used by Lillie *et al* (1941). Ceroid shares many properties with lipofuscin, although Porta (1987) suggests that newly-formed ceroid is partly soluble in lipid solvents, in contrast to lipofuscin.

The accumulation of ceroid in various tissues occurs in Vitamin E deficiency in several mammalian species. Vitamin E deficiency in the rat results in the formation of lipopigment in the duodenal lamina propria and muscle layers (Katz *et al* 1985) and in the uterus (Bieri *et al* 1980). Sarter and van der Linde (1987) reported a 'dramatic' increase in lipopigment of cerebral neurones in Vitamin E-deficient rats, but Porta (1987) found no such increase. Increased fluorescence under ultraviolet irradiation, attributed to lipopigment formation, occurs in a number of other tissues in Vitamin E deficient rats, and may be accompanied by a brown discolouration (Moore and Wang 1947). 'Brown bowel syndrome', a heavy accumulation of lipopigment in the smooth muscle of the human gastrointestinal tract found in association with chronic malabsorption and chronic hepatic disorders, is attributed to Vitamin E deficiency (Dolman and Macleod 1981, Porta 1987).

Because Vitamin E is believed to act as an antioxidant *in vivo*, the formation of ceroid in Vitamin E deficiency has led to the hypothesis that ceroid is the product of peroxidative damage to lipids. Since polyunsaturated lipids should be more susceptible to such damage than saturated lipids, a diet high in polyunsaturated fats and deficient in Vitamin E should cause increased ceroid formation. Such a diet causes a 'brown bowel syndrome'-type accumulation of lipopigment in the smooth muscle of the small intestine of dogs (Cordes and Mosher 1966). The same kind of diet has been shown to increase the fluorescence in lipids extracted from rat tissues by chloroform/methanol (Reddy *et al* 1973). However it is doubtful that the fluorescence of the lipid extracts represents lipopigment. The

emission maximum of lipid extracts reported by Reddy *et al* (1973) is not similar to those of lipopigments *in situ* (Katz *et al* 1984), and it cannot be assumed fluorescent material extracted from tissues by chloroform/methanol is derived from lipofuscin (see earlier).

Granules with the morphology, fluorescence and histochemistry of ceroid can be induced in cells in tissue culture. Such granules developed in mouse macrophages cultured in the presence of emulsions of lipid and bovine serum albumin (BSA) (Carpenter *et al* 1988). The granules formed rapidly when the lipid used was composed of polyunsaturated fatty acid esters, but did not form when mono-unsaturated fatty acid esters were used. Use of oxidized lipid to create the emulsion accelerated the accumulation of the granules, while addition of free radical scavengers had the opposite effect. Altering the proportion of protein in the emulsion had little effect. Carpenter *et al* (1988) concluded that these results support the theory that ceroid is a product of, and a marker for, lipid oxidation *in vivo*. The role of protein in ceroid formation was considered to be non-specific, possibly that of a 'vehicle' for the lipid.

Cultured human glial cells accumulate lipopigment in their lysosomes at a greater rate than glial cells *in vivo*, a difference attributed to the higher oxygen tension *in vitro* (20% compared to 3 to 4% O_2 in brain tissue). Fluorescence of cultured glial cells increased with increased oxygen tension and in the presence of Vitamin C and iron. Vitamin E and selenium, or reduced glutathione, reduced the development of autofluorescence. Thaw *et al* (1984) concluded that 'This supports the thesis that lipofuscin is derived from cellular lipid peroxidation products'. However they did not conclusively demonstrate that the fluorescence measured was an accurate measure of the lipopigment present. Furthermore the structures of the lipopigments produced *in vitro* by Thaw *et al* (1984) and Carpenter *et al* (1988) have not been defined.

Ceroid accumulates within macrophages associated with tissue necrosis in the human brain (Schroder 1980). Another lipopigment, termed haemoceroid, also forms rapidly in necrotic human brain tissue in the presence of cerebral haemorrhage. Haemoceroid granules are

distinguished from ceroid by their greater size, more intense autofluorescence, and by differences in affinity for a variety of stains, and the fact that they apparently develop extracellularly before being taken up by macrophages (Schroder and Reinartz 1980).

Induction of hepatic lipopigment formation has been reported in diseases in which metals are stored, such as haemosiderosis and Wilson's disease (Elleder 1981). Copper-associated protein granules demonstrated in human fetal liver appear to be identical to Schmorls-positive lipofuscin granules in serial sections. Neither type of granule is observed in livers of children over six months of age (Fuller et al 1990).

A number of experimental and pathological conditions exist in which the formation of lipopigment appears to be attributable to defective protein metabolism rather than peroxidation of lipids. Lipopigment accumulation has been induced in various tissues of experimental animals by the administration of inhibitors of lysosomal proteinases, such as leupeptin, an inhibitor of cystine proteases and some serine proteases. Infusion of leupeptin into the lateral ventricle of rats causes the rapid accumulation of lipofuscin-like granules in neurones (Ivy et al 1984). The leupeptin-induced pigment exhibits similar morphology and histochemistry as brain lipofuscin, and is associated with a dramatic increase in the level of dolichols, long-chain polyisoprenols associated with the lysosomal membrane (Ivy and Gurd 1988). Intravitreal injection of leupeptin results in a rapid accumulation of fluorescent inclusions in the rat retinal pigment epithelium (Katz and Shanker 1989). Intraperitoneal infusion of cystine protease inhibitors induces lipopigment accumulation in liver, kidney, and to a lesser degree pancreas, spleen, and lung of young rats and mice (Ivy et al 1990). The effect of leupeptin can be duplicated by substituting E-64C, specifically a cystine protease inhibitor, but not by using aprotinin, a serine protease inhibitor (Ivy et al 1990).

Another example of experimental lipopigment formation in vivo which appears to involve protein metabolism rather than lipid metabolism is that reported by Sharma and Manocha (1977). They found that

lipopigment could be induced in the cerebral neurones of foetal squirrel monkeys by maintaining the dams on a protein-deficient diet through most of gestation. Disturbance of protein turnover may be involved in the development of this lipopigment, which showed the morphological and histochemical characteristics of lipofuscin, and contained lysosomal enzymes. Rehabilitation of the squirrel monkeys to a protein-sufficient diet resulted in a decrease in the quantity of lipopigment in the neurones with a concurrent increase in lipopigment in perineuronal glial cells (Manocha and Sharma 1977), which supports the conclusion of Glees (1987) that microglial cells can take up lipofuscin granules from cerebral neurones (see above).

Lipopigments also occur in two types of genetic disease, the ceroid-lipofuscinoses and Chediak-Higashi syndrome, and in both cases are associated with abnormalities in protein metabolism. The group of diseases termed the ceroid-lipofuscinoses are recessively inherited diseases affecting children and some animal species, which lead to blindness, dementia, seizures, idiocy, and premature death (Zeman 1976). They are distinguished from each other principally by age of onset. In these diseases, large quantities of lipopigment accumulate in the lysosomes of neurones and a wide range of other cells. Consequently the ceroid-lipofuscinoses have been regarded as models for aging and lipofuscin formation (Armstrong et al 1983). Approximately two-thirds of the mass of lipopigment granules isolated from sheep affected by ceroid-lipofuscinosis is protein. The principal protein stored, accounting for at least 50% of the mass of the lipopigment, is the DCCD binding proteolipid subunit c of mitochondrial ATP synthase (Palmer et al 1989, 1990). This protein, which is complete and apparently unmodified, can be extracted in chloroform:methanol 2:1/100 mM ammonium acetate and is therefore classed as a proteolipid (Folch and Lees 1951). The genetic lesion in ceroid-lipofuscinosis probably relates to a defect in the degradative pathway of this protein (Palmer et al 1990, Fearnley et al 1990).

Chediak-Higashi syndrome is an autosomal recessive disease characterized by partial albinism, susceptibility to infections, and the presence in many cell types of enlarged, granular, anomalous

lysosomes, which frequently contain lipopigment. The Beige mouse is an animal model of this disease. Beige mice accumulate massive quantities of lipopigment in hepatocytes, renal tubule cells, and splenic macrophages. Exogenous protein (horseradish peroxidase, administered intravenously) cannot be demonstrated after 48 hours in the lysosomes of renal tubules of normal mice, but persists for up to 5 days in the anomalous lysosomes of renal tubules in Beige mice. The findings suggest that, in Beige mice, these lysosomes are defective in degrading protein (Essner et al 1974).

Theories of lipofuscin biogenesis

The most commonly advanced hypothesis for the biogenesis of lipofuscin is that it represents an endproduct of free radical-induced intracellular peroxidation of membrane lipids (Dolman and Macleod 1981, Lippman 1983, Nakano et al 1989). This hypothesis rests on the properties, particularly autofluorescence, which are shared by lipofuscin and ceroid, which is widely held to be a product of peroxidised lipids. By analogy, this presumed genesis of ceroid has been ascribed to lipofuscin.

The free radicals are thought to be generated by various biological processes. The initial step is believed to be the reaction of salts of transition metals such as iron, copper, and manganese with hydrogen peroxide to generate the hydroxyl radical, believed to be the most damaging molecule (Halliwell 1981, Halliwell and Gutteridge 1985).

The major source of hydrogen peroxide is the respiratory chain of the mitochondrial inner membrane (Lippman 1983), although it is also produced from the superoxide anion by superoxide dismutase. Hydrogen peroxide may initiate a direct lipid peroxidation, but is less reactive than the hydroxyl radical. Protective mechanisms exist to break hydrogen peroxide down to water, but according to the free radical theory, these mechanisms sometimes fail, allowing the generation of the hydroxyl radical (Zs.-Nagy 1988).

Free radicals, particularly hydroxyl radicals, are thought to initiate peroxidation of the unsaturated bonds of polyunsaturated

fatty acids present as components of the lipid bilayer of cell membranes. This results in the initiation of a chain reaction in which lipid free radicals are formed. Direct attack of proteins by free radicals, particularly the hydroxyl radical, is also believed to occur (Zs.-Nagy 1988). Although proteins damaged by free radicals and related oxidants are generally more susceptible to intracellular proteolysis than native proteins, extensively cross-linked proteins exhibit increased resistance to proteolysis. Oxidatively damaged proteins might undergo such cross-linking if protein damage exceeds the capacity of the proteolytic systems of the cell. This might occur during a period of unusual oxidative stress, or as a result of an age-related decline in proteolysis (Davies 1988).

Malondialdehyde, an end-product of lipid peroxidation, is highly reactive and forms irreversible cross-links with the formation of Schiff base polymers with the amino groups of a variety of macromolecules such as DNA, phospholipids, and proteins (Chio and Tappel 1969a 1969b, Zs.-Nagy 1988). The lipid peroxidation theory for the origin of lipofuscin concludes with the accumulation in the lysosome of peroxidised, polymerized residues of lipids and proteins, which are resistant to degradation by lysosomal enzymes (Brunk and Collins 1981, Zs.-Nagy 1988). There seems to be a general assumption that peroxidative damage occurs prior to the autophagy of the lipid material into the lysosome, although the literature is generally remarkably vague on the process by which free radicals from the inner mitochondrial membrane, against which protective enzymes are found in the cytosol, attack lipids in phospholipid membranes. Alternatively lipid peroxidation may occur in the lysosome, which may lack many of the antioxidative systems operating elsewhere in the cell, and which may contain metals such as Fe^{2+}, which are catalysts of lipid peroxidation (Brunk and Collins 1981). The proponents of the peroxidation theory tend to mix these two mechanisms, but they are exclusive, and have different consequences.

Cells contain protective mechanisms against oxygen-derived free radicals. Superoxide dismutase (SOD) removes superoxide, although the reaction generates hydrogen peroxide. Ascorbate can remove superoxide and the hydroxyl radical, although ascorbate also

maintains iron in the Fe^{2+} state. Hydrogen peroxide is removed by catalases and peroxidases (Halliwell 1981).

Vitamin E is believed to act as a biological antioxidant by absorbing the unpaired free radical electron thus preventing the auto-oxidative chain reaction (Sarter and van der Linde 1987). Other free radical scavengers include carotenoids, ascorbic acid, and glutathione (Halliwell and Gutteridge 1985). The lipid free radical can also be converted by an alternate pathway to a hydroperoxide, which can be converted to harmless hydroxy fatty acid in the presence of glutathione peroxidase (Donato 1981).

Various *in vitro* models of 'lipopigment' resulting from lipid peroxidation have been devised. Malondialdehyde has been shown to react readily *in vitro* with amino group-containing compounds to form highly fluorescent conjugated Schiff bases. It has been asserted that the fluorescence of lipofuscin is indicative of the presence of Schiff bases (Chio and Tappel 1969a,b, Zs.-Nagy 1988). The *in vitro* reaction of malondialdehyde with proteins produces 1,4-dihydropyridine-3,5-dicarbaldehydes, with fluorescence characteristics 'similar but not always the same as' those of age-related fluorescent substances (Kikugawa and Beppu 1987).

The *in vitro* reaction of lipid peroxidation intermediates with proteins gives rise to crosslinked polymers with strong fluorescent properties, which are insoluble in a variety of organic solvents (Chio and Tappel 1969a,b, Shimasaki *et al* 1982). Although Shimasaki *et al* (1982) claim that their studies 'indicate that most of the lipofuscin substances are oxidized fat bound tightly to protein', the excitation and emission spectra for these substances are not similar to those of lipofuscin and ceroid *in situ* as measured by Katz *et al* (1984).

Copper and iron catalyze auto-oxidation in phospholipid membranes and fatty acids *in vitro* (Gutteridge 1984). However, cells have mechanisms to maintain the free ions of metals at almost undetectable levels, and the role of metal ions in lipopigment formation has not been demonstrated under physiological conditions (Porta 1987).

The lipid peroxidation theory of lipofuscin formation is not universally accepted, and many of the reactions involved remain purely theoretical. Attempts to detect 'oxygen-centred' radicals, by electron spin resonance, in cells cultured from a dog with ceroid-lipofuscinosis were not successful (Vistnes et al 1983). No-one has conclusively identified a conjugated Schiff base with the characteristic fluorescence of lipofuscin, or demonstrated the presence of malondialdehyde in chloroform-methanol extracts of biological tissue (Brizzee and Ordy 1981). The extent of lipid peroxidation has been inferred by using the thiobarbituric acid (TBA) test to measure malondialdehyde production (Donato 1981). However the TBA test is not specific for malondialdehyde, and measuring malondialdehyde in oxidized lipids by alternative methods has shown that the TBA test gives gross overestimates of malondialdehyde production (Gutteridge 1987, Kikugawa et al 1988, Janero and Burghardt 1988, Yoden and Iio 1989, Kosugi et al 1990).

Intermediates of lipid peroxidation such as lipid peroxides have never been detected in freshly prepared tissues from undamaged organisms. There is a lack of direct experimental evidence that lipid peroxidation occurs in healthy animals, or that the rate and extent of lipid peroxidation increases in Vitamin E-deficient animals (Donato 1981). Chronic parenteral administration of large doses of Vitamin E does not ameliorate the pathological effects on the liver of iron overload in rats, although these effects are attributed to peroxidative damage to membrane phospholipids (Bacon et al 1989). The fluorescence spectra of lipofuscin are not similar to those of malondialdehyde reaction products, or those of Schiff bases (Eldred 1987). Although the enzyme glutathione peroxidase is alleged to be an important defence against auto-oxidative chain reactions in membrane lipids (Donato 1981), no deleterious phenotypic effect is observed in people who are homozygous for a gene that causes low glutathione peroxidase activity. Approximately 30% of people of Jewish ancestry, and similar proportions in other Mediterranean races, are homozygotes, in whom glutathione peroxidase activity is only half that considered 'normal'(Beutler 1979). Long-term dietary supplementation with vitamin E does not decrease the formation of

lipofuscin in rats, regardless of whether the dietary fat is saturated or unsaturated (Porta 1987).

Age pigment and the aging process

This century has seen a marked increase in life expectancy in developed countries. Furthermore there is a tendency in Western culture to view aging as a pathological process to be avoided if at all possible. As a consequence of these factors, gerontology, or the study of aging, has attracted increasing attention in recent decades. Numerous theories of aging have been proposed, incriminating virtually every type of biological molecule, and most body systems. One strongly advanced theory proposes that aging occurs as a result of lipid peroxidation as described above, and that the age pigment formed is a marker of this process (Harman 1984, 1987, 1988). Since lipofuscin is considered to be one of the most consistent and characteristic signs of cellular senescence (Dolman and Macleod 1981, Harman 1987, Porta 1987), and is believed to be the product of free radical-induced lipid peroxidation, accumulation of age pigment is considered to be strong evidence for this theory (Gordon 1974, Lippman 1983, Harman 1987). It is suggested that protective mechanisms in the cell can cope with most but not all of free radical generation and so damage occurs at a low rate (Halliwell and Gutteridge 1985). Harman goes so far as to say that 'Today it seems very likely that the assumption that there is a basic cause of aging is correct and that the sum of the deleterious free radical reactions going on continuously throughout the cells and tissues is the aging process or a major contributor to it' (Harman 1984).

Various means by which lipid peroxidation could affect cell function are proposed. Lipid peroxidation is believed to decrease membrane fluidity and to increase membrane permeability. Aldehydes produced by lipid peroxidation can react with -SH groups, causing cross-linking both within and between proteins. In consequence, enzymes requiring $-NH_2$ or -SH groups to be active might be inactivated (Halliwell and Gutteridge 1985). The significance of such damage to membranes and proteins would of course depend on whether lipid peroxidation occurs before or after the substrate enters the lysosomal system.

An increase in superoxide generation with age, and a concurrent decrease in endogenous antioxidants, have been demonstrated in houseflies by Sohal (1988). Reducing the metabolic rate of houseflies, by such methods as preventing flying and reducing the ambient temperature, results in significant prolongation of their lifespan. Increasing the metabolic rate of houseflies shortens their lifespan, increases the accumulation of 'chloroform-soluble lipofuscin-like material', and increases the concentrations of hydrogen peroxide, oxidized glutathione, and TBA-reactive material. The rate of exhalation of n-pentane, believed to be an indicator of in vivo lipid peroxidation, is also increased. A correlation between metabolic rate, intracellular oxidative stress and rate of aging is therefore surmised. While there is no experimental information on the effect of metabolic rate on lifespan in homeotherms, an inverse relationship between metabolic rate and lifespan exists in non-primate mammalian species, and hibernation prolongs the life of Turkish hamsters (Sohal 1988). However it is questionable whether lipofuscin found in mammalian cells, which is not soluble in chloroform (Elleder 1981), is comparable to the 'chloroform-soluble lipofuscin-like material' and therefore a marker of 'intracellular oxidative stress'.

Although there is a lack of direct evidence that lipid peroxidation occurs in healthy animals (Donato 1981), Gordon (1974) argues that failure to demonstrate lipid peroxides in vivo in healthy or Vitamin E-deficient animals is not contradictory of the free radical theory because lipid peroxides have a great tendency to progress beyond their 'active forms' to endproducts; specifically age pigment.

Harman (1984) found that 'dietary measures designed to decrease endogenous free radical reaction levels in mice tended to increase the average life expectancy by as much as 20% to 30%, but had little, if any, effect on maximum lifespan'. Whether these findings actually support the theory is questionable; if lipid peroxidation is 'the aging process or a major contributor to it', an increase in maximum lifespan should be observed. Other attempts have been made to modify the lifespan of animals and of cells in culture by the administration

of antioxidants, and results have been extremely variable (Halliwell 1981, Wolman 1981).

Lipopigment induced in nerve cells of young rats by various treatments including hypoxia, Vitamin E deficiency, and administration of various chemicals, remains for the rest of the lives of the rats, but the rats live a normal lifespan (Sulkin and Srevanij 1960). If age pigment and ceroid are both products of lipid peroxidation, accumulation of lipopigment in these young rats would be indicative that a large amount of lipid peroxidation had taken place. The fact that the rats live a normal lifespan appears to refute the idea that 'the sum of the deleterious free radical reactions...[is] the aging process or a major contributor to it' (Harman 1984).

Free radical reactions have been implicated in atherosclerosis (Harman 1984) and in some forms of cancer (Swartz 1984). Pryor (1984) holds the view that 'Radicals do not affect the genetically-determined maximum lifespan for each species, but radical-mediated reactions contribute importantly to those diseases that are most important in determining the extent to which a population lives to its maximum lifespan'. Age-related diseases, however, must be distinguished from the aging process (Brody and Schneider 1986, Masoro 1987).

Other theories of aging

Many theories exist which attribute aging to accidental alterations in nucleic acids. For example the somatic mutation theory postulates that random spontaneous mutations destroy genes (Curtis 1966, Kanungo 1980). Another example is the error-catastrophe theory, which proposes that errors in transcription and translation cause the accumulation of defective proteins (Orgel 1963, Kanungo 1980). In yet another theory, the cross-linkage theory, DNA is irreparably damaged by reactive molecules (Bjorksten 1974), which include free radicals (Halliwell and Gutteridge 1985) as well as a number of other biological molecules (Walford 1969).

Yet other theories attribute aging to programmed rather than accidental events, such as programmed mitotic arrest (Hayflick and Moorhead 1961, Gelfant and Grove 1974) or programmed changes in the isoenzymes of transfer RNAs and aminoacyl-tRNA synthetases (Kanungo 1980).

Reported age-related changes in DNA include decreases in methylation of DNA (Eichhorn 1983) and of histones (Thakur and Kanungo 1981) but the significance of these changes is not clear. Mammalian DNA has not been shown to be damaged, fragmented, less accurately transcribed or less accurately synthesized with age (Eichhorn 1983). The majority of studies indicate an age-related decrease in RNA synthesis (Richardson et al 1983) although there is no evidence for any change in the regions of the genome transcribed (Eichhorn 1983).

The aging process has also been attributed to changes in the immune system. Walford (1969) proposed that aging results from loss of immune self-recognition and thus increased autoimmunity. Other researchers, however, have reported evidence that the immune system, particularly cell-mediated immunity, becomes less active, rather than more active, with age (Ershler 1988, Antonaci et al 1987, Bruley-Rosset et al 1986). There is evidence that very long-lived human individuals are those that demonstrate the least decrease in immune response (Murasko et al 1986).

Other theories of aging that have been proposed include changes in isoenzymes, accidental or programmed loss of enzyme activity, and declining endocrine function (Kanungo 1980). Senescence has also been attributed to defective calcium metabolism (Selye and Prioreschi 1960), to lowered oxygen tension in tissues (McFarland 1963), and to detrimental effects upon circulation and extracellular diffusion of an age-related increase in the stiffness of collagen (Shock 1974).

Of the changes demonstrated to occur with aging, changes in enzymes and other proteins are worthy of discussion, as disturbances in protein metabolism have been implicated in the formation of some lipopigments. Proteins and protein metabolism undergo changes with age which may have some hitherto unrecognized significance in the formation of lipofuscin.

A decline in protein synthesis with age has been reported in numerous studies, but since no consistent age-related change in total tissue protein content occurs, the rate of protein catabolism must also decline (Richardson and Birchenall-Sparks 1983, Reznick et al 1985).

Age-related changes in enzymes have been extensively studied. The specific activity of many, but not all, enzymes changes with age (Kanungo 1980). The change is usually a decrease in activity. Enzymes also usually show an increased heat lability and a differing response to antiserum produced to the corresponding enzyme found in young individuals. Other properties, particularly kinetic properties, are altered slightly if at all. The altered enzymes do not show any change in net charge, N- and C-terminal residues are unchanged, and gross amino acid composition shows no differences. Phosphorylation, acylation and methylation are not involved. It is concluded that the alterations must therefore be conformational. Because protein synthesis and degradation become slowed in aged animals, enzymes remain longer in cells before replacement, so if an enzyme were unstable it might become altered (Rothstein 1983, 1985). In support of this hypothesis, Reznick et al (1985) found that antibodies prepared against purified rat renal maltase which had been thoroughly denatured in vitro could reliably identify inactive forms of maltase in senescent cells but did not recognize active enzyme forms.

Glycation

It has been known since 1912 that reducing sugars such as glucose can react with amino groups of amino acids, leading to the formation of brown cross-linked pigments. These reactions were first described by Maillard in 1912, so the collective term for reactions of this type is 'Maillard reactions' (Ulrich et al,1985). The first step in this process is nonenzymatic glycosylation (glycation), but the chemistry of the process is not well understood. Some pathological changes in diabetes, such as thickening of glomerular basement membranes, may be caused by such glycation (Garlick et al 1988). Glycation increases with age in vivo in long-lived proteins such as lens proteins and

collagen (Pongor et al 1984), and appears to cause age-related changes in bone osteocalcin (Gundberg and Gallop 1985), and lens crystallin (Ulrich et al 1985). Ulrich et al (1985) isolated and identified a fluorescent chromophore from browned, cross-linked polypeptides which had undergone Maillard reactions with glucose in vitro. The isolated chromophore is a furoyl furanyl imidazole and its structure supports the hypothesis that it is derived from the cross linking of amino groups by glucose. DNA can also undergo glycation in vitro when incubated with glucose and other reducing sugars, giving rise to stable glycation products (Krantz et al 1986, Lee 1987). The consequent damage to DNA may be the cause of the increase in plasmid mutations observed when intracellular glucose 6-phosphate levels are experimentally elevated in bacteria (Lee 1987) as well as the increased frequency of congenital abnormalities in children of diabetic mothers (Krantz et al 1986). The products of Maillard reactions share many properties with lipofuscin i.e. they are brown, fluorescent under ultraviolet light, contain protein, and accumulate with age. However, researchers studying lipofuscin have not explored the possibility that lipofuscin could be caused by nonezymatic glycation, rather than lipid peroxidation.

Concluding comments

Porta (1987) states: 'While there is no direct in vivo evidence demonstrating a link between free radical initiated lipoperoxidation and lipofuscin formation and accumulation, all the results of in vitro and in vivo studies on ceroidogenesis strongly suggest by analogy that lipofuscin is a peroxidation product of cell membrane lipids.' The point that this theory rests on analogy is an important one. Lipopigments are classified as such on the basis of observed histochemical, morphological, and fluorescent properties rather than any demonstrated similarities in chemical composition. Extensive theoretical extrapolations have been used to explain the accumulation of lipofuscin and also to develop an entire theory of aging for which that phenomenon is regarded as important evidence. However there is no direct evidence that peroxidation of lipids is a primary cause of lipofuscin formation, or is involved at all. The properties by which lipopigments are recognized do not necessarily indicate that they are

composed principally of lipid; indeed, some are clearly not derived from lipid. The 'lipopigment' stored in ovine ceroid lipofuscinosis is principally an identified protein, subunit c of mitochondrial ATP synthase, the catabolism of which is apparently defective in the disease (Palmer et al 1989,1990, Fearnley et al 1990). Derangement of protein metabolism is also implicated in other lipopigments: the protein-deficiency lipopigment induced in squirrel monkeys (Sharma and Manocha 1977), the lipopigment occurring in the Beige mouse (Essner et al 1974) and the lipopigment induced by the administration of inhibitors of lysosomal enzymes (Ivy and Gurd 1988, Ivy et al 1990). The induction of an lipofuscin-like lipopigment in mammalian tissues by the administration of leupeptin or E-64C may be evidence that the mechanism of lipofuscin formation involves perturbed activity of cystine proteinase (Ivy and Gurd 1988, Ivy et al 1990). The presence of lipids or carbohydrates could be due to failure of catabolism of proteins with which they were associated (Ivy and Gurd 1988). To attribute the biogenesis of lipofuscin to defective protein metabolism is therefore at least as valid an analogy as the lipid peroxidation theory.

To resolve the confusion, the study of the lipopigments must move beyond analogy based on a few observed characteristics, and the results of numerous peripheral experiments designed to support rather than to test hypotheses. The near complete chemical analysis of the lipopigment which accumulates in ovine ceroid-lipofuscinosis demonstrates that lipopigments are not necessarily insoluble or polymerized, as the literature on lipofuscin assumes. In order to determine whether the various lipopigments are indeed related in composition and origin, comprehensive chemical analyses need to be undertaken. To date, very few such analyses have been attempted. In particular, the nature and origin of lipofuscin can only be discovered from the analysis of lipofuscin, rather than assumed from studies of other lipopigments.

This thesis addresses these questions and focusses on the lipofuscin found in the cells of equine thyroid gland. The horse was considered to be a better model for human aging than the laboratory rats and mice commonly used in such studies, because it has the longest

potential lifespan found among the common domestic mammals, and the age at which adulthood is reached, based on skeletal evidence, is well established. Ossification of the last epiphyses to close in the horse, the secondary centres of the pelvic bones (iliac crest, tuber coxae of ilium, ischial tuberosity and acetabular part of pubis) is complete by 5 years of age (Getty 1975). In the human being, ossification of these pelvic centres is invariably complete by 23 years of age (Grant 1972). The horse may therefore be considered to be a young adult by 5 years. In contrast, it is difficult to identify the beginning of adulthood in laboratory rats and mice. Rats grow almost throughout life (Dawson 1934a), and failure of epiphyseal union has been demonstrated in strains of laboratory rats (Dawson 1929, 1934a), wild rats (Dawson 1934b), and white mice (Dawson 1934b).

The thyroid gland was chosen for the study as it is known to accumulate lipopigment (see next chapter), is readily available, and has a well-defined function. In light of earlier studies implicating perturbation of protein metabolism in the biogenesis of lipopigments (Essner et al 1974, Manocha and Sharma 1977, Sharma and Manocha 1977, Ivy and Gurd 1988, Ivy et al 1990, Palmer et al 1989,1990, Fearnley et al 1990) it was reasoned that lipopigment accumulation in the thyroid gland might be related to the metabolism of thyroglobulin. The synthesis, secretion, endocytosis, and lysosomal catabolism of thyroglobulin is the central process in thyroid function.

CHAPTER 2

THE THYROID GLAND: A REVIEW OF ITS GROSS AND MICROSCOPIC ANATOMY, PHYSIOLOGY, AND LIPOFUSCIN ACCUMULATION.

Gross anatomy

Human thyroid tissue forms a single gland composed of two lobes joined by an isthmus (Tice 1977) In contrast, the adult horse has two separate glands, because the glandular isthmus present in foals atrophies to a thin strand of fibrous connective tissue. The thyroid glands of the horse are, on average, 5 cm in length, 2.7 cm in thickness, 1.5 to 2 cm in width, and weigh 15 g each (Venzke 1975).

Histology

The functional unit of the thyroid gland is the follicle, which ranges in size from 50 to 900 um (Tice 1977) with a mean of 200 um (Doniach 1978). Each follicle is made up of a continuous single layer of thyrocytes (thyroid epithelial cells) surrounding a central extracellular pool of colloid (Fig. 4:2). Thyrocytes lie on a basement membrane under which is an extensive capillary network (Tice 1977). The height of normal human thyrocytes is 6 or 7 um (Wollman 1980) but ranges from 3 to 20 um being in direct proportion to the thyroglobulin synthesis and turnover of the cells (Doniach 1978). Thyrocytes are normally cuboidal with a basal nucleus but when the follicles are inactive the thyrocytes tend to become squamous and the mass of colloid increases. In hyperactive follicles the mass of colloid is small and the thyrocytes are columnar. Considerable variation in activity exists between follicles in the normal gland. Degenerating cells are rarely seen, but mitotic figures may be seen in hyperactive follicles (Tice 1977).

Colloid exhibits variable viscosity and may be present as a gel (Wollman 1980). Composed principally of thyroglobulin, which is a glycoprotein, colloid is positive to the periodic acid-Schiff (PAS) stain (Tice 1977), and exhibits variable eosinophilia (Doniach 1978).

Although usually homogeneous, it may contain cell debris and calcium oxalate crystals. In hyperactive thyroid, erythrocytes are frequently found in the colloid (Wollman 1980). Concentrically laminated PAS-positive structures, 5-70 um in diameter, may also be found in the colloid, particularly in very active glands and in older animals (Wollman 1980). Berthezene and Greer (1974) have shown that these structures, which they term 'Psammoma bodies', contain thyroglobulin with a lower iodothyronine content than normal thyroglobulin. Psammoma bodies may be relatively inaccessible to catabolism by thyrocytes, because the size of the structures makes endocytosis difficult (Berthezene and Greer 1974).

Normal thyroid ultrastructure

The apical plasma membrane of the thyrocyte forms numerous microvilli. At the apical end of the lateral plasma membrane there are tight junctions between cells as described by Tice (1977), and just basal to these, a ring of desmosomes encircle the cell. Interdigitations and gap junctions also occur between adjacent thyrocytes (Wollman 1980). The basal plasma membrane is usually smooth, but may be infolded in highly active glands (Wollman 1980).

The cell nucleus is oval with finely granular, partially clumped chromatin and a single nucleolus. Inactive, squamous cells have a small nucleus with condensed chromatin (Doniach 1978).

The thyrocyte cytoplasm contains a well-organized Golgi apparatus, distended cisternae of rough endoplasmic reticulum (Tice 1977), and numerous mitochondria, free ribosomes, and lysosomes (Doniach 1978). Two lysosomal populations containing different proportions of acid hydrolases can be isolated by isopycnic centrifugation, and histochemical studies of thyroid lysosomes also indicate lysosomal heterogeneity (van den Hove-Vandenbroucke 1980).

Two kinds of vesicle are found in the apical cytoplasm. Exocytotic vesicles, which transport newly synthesized thyroglobulin are approximately 0.14 um in diameter and have a matrix which usually

resembles colloid (Wollman 1980). Colloid droplets, oval membrane-bound organelles formed by macropinocytosis of luminal colloid (Tice 1977), are much larger than exocytotic vesicles, having a diameter of 1 to 1.5 um (Wollman 1980). Initially colloid droplets are slightly more electron-dense than colloid, and are acid phosphatase-negative. Older droplets have a paler matrix and are acid phosphatase-positive. Fusion with lysosomes is occasionally seen in electron micrographs (Wollman 1980).

Under normal conditions 20 times more thyroglobulin is endocytosed by micropinocytosis than by macropinocytosis in human thyroids (van den Hove-Vandenbroucke 1980). Micropinocytosis is performed through small pits that develop in the apical membrane (Wollman 1980). These form into micropinocytotic vesicles which are round, 0.06 to 0.3 um in diameter, often coated, and have an electron-density similar to, or less than, that of colloid (van den Hove-Vandenbroucke 1980).

Macropinocytosis is carried out by pseudopods of apical plasma membrane which project into the follicular lumen, sequester colloid, then fuse to form a colloid droplet which sinks into the cell (Tice 1977). This process can be recognized by light microscopy as a scalloped pattern at the periphery of the colloid. This indicates the presence of 'colloid vacuoles' ('resorption vacuoles') which are often lined with membranous strands or membrane structures resembling microvilli. In the human being, colloid vacuoles are considered to be a sign of thyroid hyperactivity (Nilsson et al 1988) but they are normal in the rat (Lupulescu and Petrovici 1968).

Thyroglobulin

Thyroglobulin is a dimeric molecule (MW 600,000) with a sedimentation coefficient of 19 S. It is made up of two identical 12 S subunits held together by disulphide bonds and also noncovalent binding forces (De Nayer and Vassart 1980). The dimeric 19 S molecule represents 90% of the total thyroglobulin in the follicle lumen, while the monomer represents 7 - 10% (van den Hove-Vandenbroucke 1980). The number of covalent bonds between the subunits increases with

iodination (De Nayer and Vassart 1980) of tyrosine residues, of which each subunit contains 67 (Malthiery and Lissitzky 1987). Primary structures of bovine thyroglobulin (Mercken et al 1985) and human thyroglobulin (Malthiery and Lissitzky 1987) have been deduced from their complementary DNA. There is evidence that the N terminal groups are blocked in the mature protein.

Thyroglobulin is 10% carbohydrate by weight. Two main carbohydrate side-chains, A and B, are linked to the polypeptide backbone through attachment to asparagine. Unit A (MW 1,800) contains N-acetylglucosamine and mannose, and Unit B (MW 2,100 to 3,300) contains these two sugars and, in addition, N-acetylneuraminic acid, fucose, and galactose. The ratio of A chains to B chains varies between species. A galactosamine-rich chain attached to serine or threonine residues by O-glycosidic bonds has been found in human and guinea pig thyroglobulin but not in bovine thyroglobulin. A fourth unit, only detected in human thyroglobulin so far, contains repeated glucuronic acid-galactosamine units attached to the peptide chain through a galactosylxylosyl-serine linkage (De Nayer and Vassart 1980).

After being manufactured by polyribosomes of the rough endoplasmic reticulum, thyroglobulin is processed through the Golgi apparatus where glycosylation takes place. Iodide is imported into the thyrocytes from plasma by an energy-dependent pumping process. Iodination of thyroglobulin is very rapid, and takes place in the exocytotic vesicles, by means of a peroxidase. Iodide is oxidised to iodine and then bound to tyrosine residues of thyroglobulin to form monoiodotyrosine (MIT) and diiodotyrosine (DIT) residues. Oxidative coupling of pairs of DIT molecules, with the extrusion of an alanine molecule, gives rise to tetraiodothyronine (T4, or thyroxine) precursors. Triiodothyronine (T3) precursors are formed by the coupling of MIT with DIT (Doniach 1978).

When exported into the colloid, thyroglobulin carries the lysosomal recognition marker mannose-6-phosphate. In a number of cell types this marker operates as a signal for targeting newly synthesised

enzyme precursors to lysosomes. It is not known why the newly synthesised thyroglobulin is not transported directly to lysosomes. Possibly a marker indicating that the protein is to be exported from the cell is also present and dominates the mannose-6-phosphate marker (Herzog *et al* 1987).

Thyroglobulin endocytosis and catabolism

Newly synthesised thyroglobulin molecules are secreted into the periphery of the follicle lumen and take several hours to diffuse, and are therefore generally the first to be endocytosed. The more iodinated molecules are preferentially endocytosed (van den Hove-Vandenbroucke 1980).

Although micropinocytosis is the major mode of endocytosis under normal circumstances (van den Hove-Vandenbroucke 1980), experimental manipulation of thyroid activity is performed using intravenous injection of thyroid stimulating hormone (TSH) which stimulates macropinocytosis. Pseudopods develop within 5 minutes in response to the injection. Colloid droplets appear after about 10 minutes. They reach a peak of about five per cell section as seen by electron microscopy after 30 minutes and slowly disappear over 2-3 hours. Primary lysosomes cluster around colloid droplets 5-10 minutes after the droplets form, but fusion is rarely observed in electron micrographs, suggesting that this is a rapid process. Fusion of a colloid droplet and lysosomes creates a phagolysosome. The half-life of thyroglobulin inside colloid droplets and phagolysosomes is estimated to be 26 minutes (van den Hove-Vandenbroucke 1980).

Several *in vivo* and *in vitro* studies suggest that thyroglobulin is not completely catabolised in the thyroid gland. *In vitro* hydrolysis of thyroglobulin by thyroid lysosomal proteases is never complete (van den Hove-Vandenbroucke 1980, Tokuyama *et al* 1987). Enzymes prepared from purified porcine thyroid lysosomes can release iodoamino acids very effectively from human and rat thyroglobulin without degrading the entire molecule to free amino acids (Tokuyama *et al* 1987), and four peptides with molecular weights of less than

4000 Daltons remain following proteolysis by this method (Tokuyama et al 1987). Small peptides account for 40 to 60% of the total degradation products of thyroglobulin in secondary lysosomes in the thyroid (Peake et al 1970). The susceptibility of thyroglobulin to hydrolysis by acid proteases in vitro is significantly increased by reduction of disulphide bonds. This reduction may be a rate-limiting step in thyroglobulin degradation in vivo (van den Hove-Vandenbroucke 1980).

The thyroid hormones, T3 and T4, are preferentially liberated compared to MIT and DIT (van den Hove-Vandenbroucke 1980). T3 and T4 diffuse through the basement membrane of the cell and the follicular basement membrane into the perifollicular capillaries. The iodine of MIT and DIT is removed by a dehalogenase. Most iodide from catabolised thyroglobulin is conserved and recycled. A trace of thyroglobulin (5 to 6 ug per litre) is present in the serum of over 70% of humans. The level is raised in hyperthyroidism and after administration of TSH (Doniach 1978).

Regulation of thyrocyte activity

The activity of thyrocytes is mediated through TSH secreted by the pituitary gland, which in turn is stimulated by the release of TSH-releasing hormone (TRH), from the hypothalamus (Demeester-Mirkine and Dumont 1980). Elevated blood T3 and T4 levels suppress TSH secretion while iodine deficiency and low levels of T3 and T4 stimulate production. TSH stimulates endocytosis of colloid and synthesis of both thyroglobulin and thyroperoxidase. The action of TSH is rapid, although all follicles do not respond at the same rate (Tice 1977). The progressive recruitment of thyrocytes with a gradually increasing threshold for responsiveness to hormonal stimulation is thought to allow a controlled and flexible response of the gland as a whole. Evidence exists for similar stepwise recruitment in other endocrine tissues (Gerber et al 1987).

TSH secretion in man exhibits circadian periodicity, with maximum values found during an evening peak immediately before sleep

(Condliffe and Weintraub 1979, Demeester-Mirkine and Dumont 1980). In addition to the circadian periodicity there are brief, low amplitude bursts of TSH secretion throughout the day and night (Condliffe and Weintraub 1979), with a periodicity of 1 to 3 hours (Hershman et al 1976).

The membrane of exocytotic vesicles contributes to the area of apical plasma membrane available for the formation of colloid droplets. Exocytosis of newly synthesised thyroglobulin precedes macropinocytosis of colloid, and the magnitude of the cell response to TSH is directly related to the pool of exocytotic vesicles available in the cell (van den Hove-Vandenbroucke 1980).

Thyroid hyperplasia can be induced in the mouse by an iodine-deficient diet or by the use of a goitrogen, such as propyl-thiouracil (PTU), which inhibits iodide oxidation and binding. Administration of iodide reverses thyroid hyperplasia. A high dose of iodide (10-100 ug) to mice has a direct toxic effect on hyperplastic thyroid tissue, resulting in abundant necrosis and inflammation within a few hours, and the appearance of lipofuscin granules within 12 hours in surviving thyrocytes, follicular lumina, and macrophages. Involution of thyroid hyperplasia without inflammation, necrosis and lipofuscin accumulation can be induced by the administration of a moderate dose of iodide (1 ug/day) or by administration of T3 (1 ug/day). Using these methods, reduction in cell numbers occurs by apoptosis and excessive membrane area is lost by shedding into the follicular lumina (Mahmoud et al 1986).

Lipofuscin in thyrocytes

There have been numerous reports of lipofuscin in thyroid tissue in the literature, although these have been largely descriptive, and the current study represents the first attempt at comprehensive analysis of this lipofuscin.

The spontaneous formation of lipofuscin in thyrocytes has been reported in man (Heimann 1966, Lupulescu and Petrovici 1968, Klinck

et al 1970, Mizukami and Matsubara 1982, Matsubara et al 1982, Delprado and Carter 1984, Gordon et al 1984, Alexander et al 1985, Landas et al 1986, Ohaki et al 1986), cats (Ives et al 1975), dogs, monkeys, guinea pigs (Gordon et al 1984), and horses (Anderson and Capen 1978). Accumulation of lipofuscin in thyrocytes causes gross darkening of the tissue with age (Capen 1985). Lipofuscin accumulation is increased in the thyroids of humans with mucoviscidosis (Borel and Reddy 1973), and a similar pigment has been induced by the administration of the antibiotic minocycline in man (Reid 1983, Delprado and Carter 1984, Gordon et al 1984, Alexander et al 1985, Landas et al 1986, Ohaki et al 1986), rats (Gordon et al 1984, Tajima et al 1985), dogs, and monkeys (Gordon et al 1984). It is debated whether the pigment induced by minocycline is lipofuscin (Reid 1983, Delprado and Carter 1984, Wajda et al 1988), neuromelanin (Landas et al 1986), a reaction product of minocycline with iodine or thyroid peroxidase (Tajima et al 1985, Senba et al 1988, Ohaki et al 1986), or a combination of pigments (Gordon et al 1984, Alexander et al 1985).

Lipofuscin was found in all specimens in an ultrastructural study of normal thyroid tissue from 21 persons aged 14 to 74 years (Klinck et al 1970). Two studies of thyroid lipofuscin in consecutive human autopsies, covering a total of 750 cadavers, showed that although lipofuscin quantity generally increased with age, a small proportion of individuals showed no lipofuscin deposition even in their eighth or ninth decade (Matsubara et al 1982, Ohaki et al 1986). Lipofuscin rarely occurred below the age of 15 years (Matsubara et al 1982).

Thyroid lipofuscin has been described as yellow-brown (Alexander et al 1985, Ohaki et al 1986) and as dark brown (Reid 1983). Gordon et al (1984) reported that human thyroid lipofuscin was fluorescent but Reid (1983) found fluorescence in the lipofuscin of only one of three naturally pigmented human thyroids.

Human thyroid lipofuscin was found to be acid-fast with the Zeihl-Neelsen stain (Landas et al 1986, Gordon et al 1984) although the length of the Zeihl-Neelsen reaction was not specified. It was also

shown to be acid-fast with the Armed Forces Institute of Pathology (AFIP) stain for lipofuscin (Ives et al 1975, Reid 1983), which is a modified long Zeihl-Neelsen method. The reaction to Schmorl's stain has been reported as positive (Gordon et al 1984) and also as negative (Alexander et al 1985), while the reaction to periodic acid-Schiff (PAS) has been reported as positive (Landas et al 1986), as well as negative (Gordon et al 1984), and as 'red-brown' (Alexander et al 1985). Lipofuscin in frozen thyroid sections has been reported as osmiophilic, sudanophilic (Gordon et al 1984), and Oil Red O positive (Reid 1983).

In 9 out of 15 biopsy samples from human volunteers, occasional lipofuscin granules were iron-positive using the Turnbull blue stain (Gordon et al 1984) and Alexander et al 1985) demonstrated that thyroid lipofuscin was 'focally positive' for iron. Gordon et al (1984) and Landas et al (1986) found that thyroid lipofuscin was positive to Masson's Fontana silver stain, but Alexander et al (1985) reported that it was negative. Landas et al (1986) found that pigment in the same sections of human thyroid could be divided into Fontana-negative pigment that was resistant to permanganate/oxalic acid sequence bleach for melanin and Fontana-positive, bleach-labile pigment. They concluded that the pigments were lipofuscin and neuromelanin respectively.

Lipofuscin granules were one of the three most conspicuous structures, along with mitochondria and rough endoplasmic reticulum, observed in human thyroid cytoplasm by electron microscopy. They occupied up to half of the visible cytoplasm in a section of a thyrocyte, and sometimes indented the cell nucleus (Klinck et al 1970). Their ultrastructural characteristics have been studied in humans (Klinck et al 1970, Heimann 1966, Lupulescu and Petrovici 1968, Alexander et al 1985) and in cats (Ives et al 1975). Thyroid lipofuscin granules were irregular in shape (Heimann 1966, Lupulescu and Petrovici 1968, Alexander et al 1985) and varied considerably in size. Diameters ranged between 0.2 and 4 um (Heimann 1966, Matsubara et al 1982). It has been suggested that granules may increase in size by fusion (Ives et al 1975).

Each granule was made up of two components, a matrix which is usually very electron-dense (Lupulescu and Petrovici 1968, Alexander et al 1985)and one or more electron-lucent circular structures embedded in it (Heimann 1966, Lupulescu and Petrovici 1968, Klinck et al 1970). In thyroid lipofuscin of the domestic cat, the electron-dense areas became more dense and coarsely granular with age, and vacuolation increased. Membranous and lamellated inclusions have been described in the electron-lucent areas (Ives et al 1975).

The nature of the contents of the electron-lucent areas is unclear. Most studies describe these areas as lipid (Borel and Reddy 1973, Ives et al 1975, Matsubara et al 1982, Alexander et al 1985) because of their appearance but there are no biochemical analyses to support this conclusion and it has been questioned (Heimann 1966). Lupulescu and Petrovici (1968) identified these areas as colloid droplets.

Alexander et al (1985) described thyroid lipofuscin granules as membrane-bound, but other studies have reported that they lacked a distinct membrane (Heimann 1966, Delprado and Carter 1984) although they did have a very dense peripheral zone of varying thickness (Heimann 1966).

Thyroid lipofuscin granules demonstrated both acid phosphatase and beta glucuronidase activity, indicating lysosomal function. Small dense lysosome-like vesicles were often found in close proximity to the granules. Colloid droplets sometimes contained particulate inclusions resembling lipofuscin granules, and may be transformed into them (Ives et al 1975).

Comparison of serial sections of murine thyroid by electron microscopy and analytical ion microscopy suggested that cytoplasmic patches of iodine correspond to lipofuscin granules and secondary lysosomes. Iodine in these cytoplasmic sites had a lower turnover rate than that in the colloid, and may be excluded from hormone synthesis (Mestdagh et al 1990).

Iodine was demonstrated by energy-dispersive x-ray analysis in the thyroid lipofuscin of a dog, a guinea pig, a squirrel monkey and 16 humans. The level of iron measured by the same method was higher than that of the surrounding cytoplasm in the animals, and occasionally in the humans, while a peak for potassium was observed in the animals but not the humans. A calcium peak was also demonstrated in the lipofuscin of the monkey (Gordon et al 1984).

The presence of metals in thyroid lipofuscin may be a reflection of its lysosomal nature. Lysosomes sequester a number of metals including iron (Hanaichi et al 1984, LeSage et al 1986, Trump et al 1973), copper (Hanaichi et al 1984), aluminium (Gruca and Wisniewski 1984), platinum (Seidel et al 1985), gold, mercury, lead and tellurium (Sternlieb and Goldfischer 1976). Experimental loadings with some of these metals are associated with the appearance of lysosome-derived lipofuscin-like bodies containing the metals in hepatocytes (Sternlieb and Goldfischer 1976).

Other age-related changes in the thyroid gland

At birth human thyroid follicles are small, contain weakly eosinophilic colloid and are lined with columnar cells. During childhood the colloid becomes more abundant and more eosinophilic, and the thyrocytes become cuboidal. From about 12 years the follicles show increasing variability in size and shape (Doniach 1978). An age-related increase in variability of follicle size has also been observed in other species (Wollman 1980). In elderly humans the follicles are small, the colloid is 'broken up', and the thyrocytes are cuboidal (Doniach 1978).

'Cold' follicles, characterised by large size, intensely PAS-positive colloid and squamous thyrocytes, develop in mouse thyroid with age. They first appear at 5 months and may account for 60 to 80% of follicles by 13 months of age. Probably as a consequence of cold follicle development, the thyroids of old mice are twice the size of those of young mice and contain twice as much thyroglobulin, but the total number of cells and follicles does not increase. Cold

follicles can be prevented by lifelong TSH stimulation. Thyrocytes of cold follicles are poorly responsive to TSH, and defective in endocytosis and iodination of thyroglobulin. Turnover of colloid is greatly slowed (Studer et al 1978, Gerber et al 1987).

The formation of densely basophilic granules in follicular lumina occurs frequently in aged dogs and occasionally in other species. It has been suggested that these granules are formed by the precipitation of calcium on aggregated colloid, which may have abnormal chemical structure. They develop in animals with normal blood calcium and phosphorus levels and do not appear to be consistently associated with other thyroid diseases (Capen 1985).

Calcium oxalate crystals appear in the colloid during human childhood and become more numerous with age (Doniach 1978, Reid et al 1987). They were found in 79 of 100 routine consecutive human autopsies. Development of crystals is exacerbated by diffuse hyperplasia, but usually decreased in adenomas and carcinomas. (Reid et al 1987). Calcium oxalate crystals were found in three of five horse thyroids examined and in the thyroids of five sheep, but were not found in the small numbers (five and two respectively) of bovine and canine thyroids examined (Hackett and Khan 1988). The aetiology and pathogenesis of deposition of calcium oxalate crystals are unknown, although there is a high concentration of calcium in thyrocytes and in the colloid (Doniach 1978).

CHAPTER 3

GENERAL MATERIALS AND METHODS

Histology.

Horses. Tissue for histology was obtained from 37 horses, which ranged in age from a stillborn premature foal (>300 days) to a 35 year old pony. Most horses were killed by a shot to the head at Hunt Club Kennels in the Manawatu region. A small number were killed by barbiturate overdose for post-mortem examination in the Veterinary Faculty, Massey University. The horses were killed for reasons other than systemic or endocrine disease. The age of each horse was obtained from the owner or from any age-brands present, and in some cases an experienced horseman also examined the dentition of the horse and estimated its age on that basis. Both thyroid glands were excised and kept on ice until fixed within 30 minutes of death.

Each horse was given a code number for histological study, consisting of the age of the horse followed by a letter to distinguish horses of the same age (Table 3:1). Exceptions were the premature foal, identified as PR, and an 8 month old foal identified as 8M.

Table 3:1. Horses used for histology.

Age	Histology code
premature	PR
8 months	8M
1 year	1A
3 years	3A, 3B
5 years	5A
6 years	6A, 6B
7 years	7A
9 years	9A, 9B
12 years	12A, 12B, 12C, 12D
15 years	15A, 15B, 15C
20 years	20A, 20B, 20C, 20D, 20E, 20F
25 years	25A, 25B, 25C, 25D, 25E, 25F, 25G
28 years	28A, 28B
30 years	30A, 30B
32 years	32A
35 years	35A

Fixation and light microscopy. Sections of paraffin-embedded thyroid tissue from 37 horses were studied. A 5 mm slice of tissue was removed from one pole of one gland, and a further 5 mm slice from the exposed tissue fixed in 10% formol saline for 48 hours.

Serial sections of paraffin-embedded tissue were stained with Haematoxylin and Eosin (H&E), Periodic Acid-Schiff (PAS)(Culling 1974), and Schmorl's stain (Stevens 1968), and a section left unstained for fluorescence microscopy. Other stains used on some sections were the Armed Forces Institute of Pathology (AFIP) stain for lipofuscin (Luna 1968), Masson's Fontana (Culling et al 1985), Perl's stain for iron (Stevens 1968), Feulgen-Naphthoic acid Hydrazine (Feulgen-NAH) (Culling et al 1985), Methyl Green-Pyronin (MGP)(Culling et al 1985), and von Kossa's stain (Culling et al 1985).

Statistical analysis. Data were analysed by linear regression. The accuracy of the model was analysed by calculation of the coefficient of determination, R^2, defined as the proportion of the variation in the values of the dependent variable that is 'explained' by variations in the values of the independent variable (Cangelosi et al 1983).

Concanavalin A labelling. Sections were stained with Concanavalin A/Peroxidase according to the following method, adapted from those of Avrameas et al (1976), and Faye and Chrispeels (1985). Sodium chloride, 500 mM, and Tris(hydroxymethyl)aminomethane (Tris), 20 mM, were dissolved in deionised water, and pH adjusted to 7.4 with concentrated HCl to make Tris-buffered Saline (TBS). TTBS (Tween/TBS + cations) was prepared by the addition of 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.1% v/v polyoxyethylene-sorbitan monolaurate (Tween 20) to TBS. Deparaffinised sections were incubated in 3% hydrogen peroxide (H_2O_2) in TBS for 15 minutes at room temperature, washed 3x in TTBS, then incubated for 15 minutes at room temperature in TTBS in which was dissolved 100 ug/ml Concanavalin A, and washed 3x in TTBS. Sections were then incubated for 15 minutes at room temperature in TTBS in

which was dissolved 50 ug/ml horseradish peroxidase. Sections were washed 3x in TTBS, followed by 1 wash in TBS, then immersed in staining solution, prepared immediately before use by dissolving 60 mg 4-chloro-1-naphthol in 20 ml of 100% methanol, and adding 80 ml TBS and 60 ul of 60% H_2O_2. Serial sections were developed for 0.5, 1, 2, 4, and 8 minutes. After developing, sections were washed 2x in distilled water, air-dried, and coverslips were applied with glycerine jelly.

Fluorescence microscopy. Fluorescence was examined in unstained sections, using a Zeiss Axiophot microscope (Zeiss, Heidelberg, Germany), barrier filter 590 nm, exciter filter 510-560 nm, and dichroic beam splitter 580 nm.

Frozen sections. Thyroids were collected immediately after euthanasia and frozen sections cut within 90 minutes of death. They were stained with Sudan Black B (Culling et al 1985) and Oil Red O (Culling et al 1985).

Electron microscopy. Electron microscopy was performed on thyroid tissue from 10 horses, aged 9 to 35, from the group used for the light microscopy study. Tissue was fixed as soon as possible after death in modified Karnovsky's fixative (Karnovsky 1965) of 2% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.3 held at 4°C. Unless otherwise stated the tissue was then post-fixed in 1% osmium tetroxide for 1 hour. Tissue was then dehydrated in graded alcohols and propylene oxide and embedded in Durcupan^R ACM epoxy resin (Fluka Chemie AG, CH-9470 Buchs, Switzerland). Sections were cut at 60-80 nm and, unless otherwise stated, stained with uranyl acetate and lead citrate.

Production of antibody for immunocytochemistry. Equine thyroglobulin, purified and supplied by R. Martinus of this laboratory, had been isolated from 50 g of equine thyroid by ammonium sulphate fractionation (Weirsinga and Chopra 1982) and further purified by high performance liquid chromatography on a TSK 3000 SW Analytical column eluted with 0.15 M sodium chloride. This thyroglobulin was

dissolved, 1 ug/ul, in saline and emulsified with an equal volume of Freund's complete adjuvant by the twin-syringe technique (Goding 1983). Four adult New Zealand White rabbits were inoculated intramuscularly 600 ul emulsion containing 300 ug equine thyroglobulin. Booster injections of the same quantity in Freund's incomplete adjuvant were given 5 weeks after the first inoculation. After a further 4 weeks the rabbits were bled by cardiac puncture, the serum separated by centrifugation for 10 minutes at 1500 g max at room temperature, and stored at -20^{o}C. The production of anti-thyroglobulin was assayed by double diffusion in agarose (Ouchterlony technique as described by Wang 1982) and by Western blotting and immunostaining (see below). All of the rabbits developed antibody against equine thyroglobulin.

Electroblotting and immunostaining. Equine thyroglobulin was transferred from a nondenaturing 5% polyacrylamide gel to polyvinylidene difluoride (PVDF) membrane (Immobilon^{TM}, Millipore Corporation, Bedford, MA) in a 2005 Transphor electroblotting apparatus (LKB, Bromma, Sweden), according to the method of Matsudaira (1987) with the following modifications: The transfer buffer was Tris HCl, 25 mM, and Glycine, 150 mM, in deionised water, and the transfer was for 18 hours at 0.5 mA. Electroblotted protein was by stained with 0.2% amido black (Wilson 1979). Thyroglobulin was detected on parallel unstained blots with the anti-thyroglobulin antibody by the following technique.

Phosphate buffered saline-Tween (PBST) medium was prepared by the addition of 0.1% v/v Tween 20 to phosphate-buffered saline solution, pH 7.3, containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4. Substrate solution was made by the addition of 2 ml of a solution of 4-chloro-1-naphthol in methanol, 3 mg/ml, and 13 ul 60%H_2O_2 to 10 ml of 50 mM Tris HCl and 171 mM NaCl in water. The PVDF membrane was wet with 100% methanol and immediately immersed for 5 minutes in deionised water, washed 3x10 minutes in PBST, and incubated in a solution of 5% BSA (Immunochemical Products Ltd) in PBST for 90 minutes at room temperature. The membrane was washed 3x10 minutes in PBST, incubated in rabbit serum diluted 1/10 in PBST

for 2 hours at room temperature, then 4x10 minutes in PBST. The membrane was incubated in peroxidase conjugated goat antirabbit IgG (Immunochemical Products Ltd) diluted 1/500 in a solution of 1% BSA in PBST for 2 hours at room temperature, washed 4x10 minutes in PBST and shaken horizontally in substrate solution until the blue-labelled protein was visible. The presence in rabbit serum of antibody to the blotted protein was confirmed. The membrane was then rinsed in water, dried and stored in the dark.

Immunogold labelling of equine thyroid tissue. Thyroid tissue, surgically removed from an aged mare under general anaesthesia, was cut into cubes approximately 1 mm^3 and fixed for 1 hour at room temperature in 2% glutaraldehyde in 0.05 M cacodylate buffer pH 7.2. The tissue was dehydrated then infiltrated with Lowicryl K4M (Chemische Werk Lowi) resin according to the method of Wells (1985) using a liquid nitrogen-controlled low temperature chamber. Samples were embedded in capsules in fresh resin and polymerised in a 360 nm UV light box for 24 hours at -20^{o}C and then at room temperature for 24 hours.

Thin sections were mounted on grids and incubated for 1 hour at 37^{o}C with anti-equine-thyroglobulin rabbit serum (see above) diluted 1/100 with 0.1 M phosphate buffer pH 7.3 containing 1% Tween 20 and 1% gamma globulin-free bovine serum albumin. They were then washed with 5 drops of 0.1 M phosphate buffer pH 7.3 containing 0.1% Tween 20 and incubated for 1 hour at 37^{o}C with Jannsen Auroprobe 15 nm gold labelled goat anti-rabbit IgG diluted 1/20 with the same buffer as that used to dilute the rabbit serum. Grids were washed again with 5 drops of 0.1 M phosphate buffer pH 7.3 containing 0.1% Tween 20, followed by 5 drops of distilled water, and stained with uranyl acetate and lead citrate.

Isolation and analyses of lipofuscin.

Horses. Lipofuscin was extracted from thyroid glands obtained from healthy horses. These horses ranged from 2 to 40 years old, and were from diverse backgrounds within the lower half of the North Island of

New Zealand. In each case, the owner's estimate of the age of the horse was obtained, and an experienced horseman examined the dentition of the horse to verify this. Pairs of thyroid glands were removed immediately after the horse was killed by a shot to the head, and transported on ice. Lipofuscin was extracted within 24 hours of death.

Isolation of lipofuscin. Thyroid glands were sliced into 5 mm slices and the capsule removed. Obvious gross lesions such as adenomas or cystic follicles were excised and discarded. The tissue, 25 g, was homogenised in 100 ml phosphate-buffered saline solution, pH 7.3, containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, in a Kenwood (Kenwood Manufacturing Co. (NZ) Ltd., Auckland, New Zealand) food processor with mincing blades fitted, at speed "3" for 5 minutes. Homogenisation was completed in a Sorvall Omnimixer (Ivan Sorvall Inc.) for 45 seconds at full speed. The pellet obtained after centrifugation in a swing-out rotor for 30 minutes at 1500 g max at 4°C in a GLC-1 centrifuge (Ivan Sorvall Inc.) was resuspended in 25 ml deionised water and sonicated for 2 minutes at full speed in a sonicator (MSE Soniprep 150 Ultrasonic Disintegrator) using the small probe assembly. The suspension was filtered through glass wool, and a pellet obtained by centrifugation for 10 minutes at 4500 g max at 4°C. This pellet was resuspended in 20 ml of deionised water, sonicated and a pellet obtained as before. The final pellet was resuspended in 10 ml deionised water. A sample was also fixed and processed for electron microscopy.

Density determination. The isopycnic densities of isolated lipofuscin granules were determined by centrifugation on 1 M to 3.4 M caesium chloride gradients in a Beckman Ultracentrifuge SW 25 rotor at 70,000g max for 16 hours at 4°C. Some gradients were centrifuged for 56 hours to ensure that isopycnic conditions had been reached. Fractions were collected from the highest to lowest density and monitored by 280 nm absorbance and electron microscopy.

Amino acid analysis. Lyophilised lipofuscin, 0.6 to 10 mg, was hydrolysed *in vacuo* in 6 M HCl containing 0.1% phenol at 110°C for 24 hours, the hydrolysate evaporated to dryness over NaOH *in vacuo*.

Quantitative amino acid analysis of the hydrolysate was performed on a Beckman 119 BL amino acid analyser using the standard protein hydrolysate program. Sodium citrate buffers were used as follows:

Buffer A	pH 3.53 0.20 N Na^+	21 minutes
Buffer B	pH 4.12 0.40 N Na^+	19 minutes
Buffer C	pH 6.40 1.00 N Na^+	77 minutes

Hydrolysates from nine horses were analysed for furosine by prolongation of elution time in buffer C by 30 minutes and comparison with a furosine standard.

Tryptophan analysis. Tryptophan estimations were carried out by an adaptation of the method of Nielsen and Hurrell (1985). Lithium hydroxide (LiOH), 4.3 M, was added at a rate of 0.2 ml per mg of lyophilised lipofuscin and the samples incubated *in vacuo* 18 hours at 110°C. After cooling, the samples were titrated to neutral with concentrated HCl, filtered through hydrophilic filters, 0.45 um, with measured aliquots of deionised water. A known volume of this solution was then analysed and compared with a tryptophan standard.

Soluble protein extraction. An equal volume of an aqueous solution containing 6% w/v lithium dodecyl sulphate (LDS) and 20% v/v 2-mercaptoethanol was mixed with lipofuscin suspended in deionised water. The mixture was incubated at 90°C for 5 minutes and the insoluble fraction pelleted by centrifugation for 10 minutes at 4500 g max at 4°C. The supernatant containing soluble proteins was stored at -20°C.

Lithium dodecyl sulphate-Polyacrylamide gel electrophoresis (LDS-PAGE). Soluble proteins were separated by LDS-PAGE according to the method of Laemmli (1970) on 15% gels with a bisacrylamide to acrylamide ratio of 1:29 w/w. Sucrose, 10%, was added to the main gel. Molecular weights of proteins were determined by the comparison of their migration rates with those of molecular weight standards (Sigma, Kit No. MW-SDS 70 L, as corrected by Kratzin *et al* 1989).

Silver staining of gels. Gels were fixed for 1 hour in 12% w/v trichloroacetic acid and then silver stained as described by Fearnley

et al (1990). Steps in silver staining and overstaining procedures from the addition of silver nitrate solution to the completion of fixing in 5 % acetic acid solution were carried out in a darkroom under a photographic safelight (Kodak, Wratten Series filter OB). After the gel was washed for 3x 10 minutes in deionised water, the stain was decolourised in 200 ml Farmer's Reducer solution (30 mM potassium ferricyanide ($K_3Fe(CN)_6$) and 65 mM sodium thiosulphate ($Na_2S_2O_3.5H_2O$)). The Farmer's Reducer was removed from the gel by sequential washes in deionised water until all yellow colour had disappeared. The gel was then agitated gently for 30 minutes in 0.25% silver nitrate solution, rinsed for 2 minutes in deionised water, and overstaining developed with a solution of 0.28 M sodium carbonate and 0.5 ml/1 formaldehyde. Developer was added in 200 ml aliquots. The first aliquot was replaced after 1 minute. Subsequent washes in developer, for not more than 4 minutes each, were carried out until the stain had developed. The development was stopped with a solution of 5% acetic acid for 5 minutes. Gels were stored sealed in plastic bags with 10 ml 5% acetic acid solution to prevent dehydration.

Electroblotting from gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon^{TM}, Millipore Corporation, Bedford, MA) in a 2005 Transphor electroblotting apparatus (LKB, Bromma, Sweden) according to the method of Matsudaira (1987) for 3 hours at 0.4 mA. After electroelution PVDF membranes were washed in deionised water for 2-3 minutes, stored dry at 4^{o}C or stained immediately. Protein bands were stained with 0.2% amido black (Wilson 1979) or stained with 0.2% Coomassie Brilliant Blue R-250 in 50% methanol containing 10% acetic acid for 2-5 minutes, and then destained in 50% methanol containing 10% acetic acid for 5-10 minutes at 25^{o}C.

Protein sequencing. Sequencing of proteins blotted onto PVDF membrane was attempted by J.E Walker, J.M. Skehel, and I.M. Fearnley at the M.R.C. Laboratory of Molecular Biology, Hills Road, Cambridge, UK. Coomassie Blue-stained protein bands of interest were cut from the PVDF membrane and placed in the cartridge block of a 470A gas phase sequencer, in the presence of only the Teflon seal, with neither polybrene nor glass fibre filter discs.

Lectin affinity labelling of electroblotted proteins. Lectin affinity labelling of glycoproteins blotted onto PVDF matrix was attempted with Concanavalin A-Peroxidase according to the method of Faye and Chrispeels (1985), using 20% methanol in the staining solution.

Proteolipid analysis of equine thyroid lipofuscin. Fresh lipofuscin was resuspended to a volume of 1 ml in deionised water and mixed with 40 ml of a 2:1 solution of Chloroform:methanol containing 100 mM NH_4OAc. The suspension was filtered through glass wool into a separating funnel, 10 ml 100 mM solution of tetrasodium EDTA was added to the filtrate, and the solutions were mixed and allowed to separate in the dark for 24 hours. The material retained by the glass wool was rinsed once with ethanol and once with deionised water, then collected, lyophilised, and weighed. A sample of this was hydrolysed for amino acid analysis, and a further sample was separated by LDS-PAGE. A small quantity of interfacial material formed during the separation of the aqueous-soluble and chloroform-soluble fractions. A sample of this was hydrolysed for amino acid analysis, and a further sample was separated by LDS-PAGE. The chloroform phase was evaporated to a volume of 1 ml, and 9 ml diethyl ether was added. The precipitate which formed at -70^{o}C over 10 days was pelleted at 1500 g for 15 minutes. A sample of this was hydrolysed for amino acid analysis, and a further sample was separated by LDS-PAGE.

Elemental analysis. Samples of lyophilised lipofuscin (20 mg) that had been washed by centrifugation (x3) in distilled water were analysed for C, H, N, O, S, and total halide content by the Microanalytical Laboratory, Chemistry Dept, University of Otago.

Lyophilised lipofuscin was ashed in borosilicate test tubes at 500^{o}C for 16 hours, and the ash dissolved in 2 M HCl. Quantitative analysis, carried out in an Applied Research Laboratories ARL 34000 plasma emission spectrometer, was obtained of B, Na, Mg, Al, P, K, Ca, Cr, Mn, Fe, Ni, Cu, Zn, Sr, Mo, Cd, and Pb.

Lipid extraction and analyses. Lipids were extracted from fresh thyroid tissue and freshly isolated lipofuscin, according to the method of Folch et al (1957). The extraction solvents contained 0.05% w/v butylated hydroxytoluene (BHT). Extracted lipids were dissolved in cyclohexane, sealed in glass vials under nitrogen and stored in the dark. Lipids were extracted from equine liver, ovine liver, ovine adipose tissue, and hen egg yolk by the same method for use as standards. All lipid extractions were carried out as quickly as possible with exposure to sunlight and heat avoided.

Phospholipids were separated by thin-layer chromatography of total lipid samples on silica gel (Skipski et al 1964) and stained with iodine vapour. Quantitative analysis of phospholipids isolated by acetone precipitation (Kates 1972) was attempted using high-field ^{31}P nuclear magnetic resonance (NMR) spectroscopy according to the method of Sotirhos et al (1986).

Neutral lipids were isolated by acetone precipitation of the phospholipids from total lipid extracts (Kates 1972) and separated by thin layer chromatography (TLC) on silica gel sheets (Bakerflex 1B, J. T. Baker Chemical Co., Phillipsburg, NJ.) developed with hexane : ether : acetic acid; 80 : 20 : 1, and stained with iodine vapour. Neutral lipids were characterised by co-migration of standards and the neutral lipids of hen egg yolk.

Dolichol and cholesterol concentrations were obtained by normal phase high-performance liquid chromatography (HPLC) on a cyanopropyl column according to the method of Palmer et al (1984), with the modification that the eluant used was 0.4% isopropanol in hexane.

DNA concentration. Colourimetric estimation of DNA concentration in lyophilised lipofuscin was performed using the diphenylamine procedure (Burton assay, Burton 1956) according to the method of Richards (1974), with the variation that each sample in 2.5 ml of 15% perchloric acid was incubated at room temperature for 2.5 hours. This incubation was carried out because of the relatively insoluble

nature of the lipofuscin. A standard curve was obtained using herring sperm DNA, 4 to 40 ug.

DNA characterisation. Nucleic acids were extracted from fresh equine thyroid lipofuscin and from leucocytes of the same animal, a 7 year old mare. To minimise fragmentation of nucleic acids, the sonication steps were omitted from the lipofuscin isolation. Blood was collected by jugular venipuncture into 10 ml Vacutainer tubes (Becton Dickinson) containing potassium EDTA anticoagulant. The leucocyte layer (buffy coat) was separated by centrifugation for 10 minutes at 1500 g at room temperature, and washed of erythrocyte contamination with 5 ml of phosphate-buffered saline (PBS) solution, pH 7.3., containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4. The centrifugation was repeated and the pellet resuspended in deionised water.

Nucleic acids were extracted from the lipofuscin or leucocytes in 10 ml aqueous solution by phenol extraction (Mew *et al* 1985). The concentration of nucleic acid in the solution after dialysis was measured by spectrophotometry according to the method of Brenner *et al* (1982) in a Cecil 599 spectrophotometer and DNA concentration calculated by the formula:

DNA concentration (mg/ml) =

$$\frac{OD_{258nm} - OD_{300nm}}{20} \times \text{dilution factor}$$

Agarose gel electrophoresis and photography were performed according to the method of Mew *et al* (1985) with the variation that the gel was 1.4% agarose. Complete lambda DNA and restriction endonuclease fragments of it were used as molecular weight markers. Restriction endonuclease digestion of DNA was performed using the restriction enzymes *DraI*, which cleaves 5′-TTT^{V}AAA-3′, and *MspI*, which cleaves 5′-C^{V}CGG-3′.

To aliquots of nucleic acid, 13.2 ul (2.3 ug) from equine leucocytes, or aliquots of lipofuscin nucleic acid, 11.2 ul (2.7 ug), were added

3 ul of REact^{TM}1 reaction buffer, 4 ul DraI (10 units/ul) or 4 ul MspI (12 units/ul). Digestion was performed according to the method of Mew et al (1985) and electrophoresis carried out on a 1.4% agarose gel.

Phenol-Sulphuric acid assay for neutral sugars (Beeley 1985). Triplicate standards of mannose, 0 to 60 ug, were used to construct a standard curve. The sample was mixed with 0.5 ml distilled water. An equal volume of 5% phenol in water was added, and the solutions mixed. Concentrated sulphuric acid (AnalaR, BDH), 3 ml, was added rapidly and immediately mixed by vortexing. The tubes were allowed to cool and the absorbance read at 490 nm within 1 hour.

Sample preparation for gas-liquid chromatography of sugars. Lyophilised lipofuscin, between 2 and 10 mg, was hydrolysed in 2 M HCl in vacuo at 100^{o}C for 1, 3 and 5 hours. The sample was then evaporated to near dryness and dried in vacuo over NaOH pellets. The samples were resuspended in 5 ml water and the lipids extracted according to the method of Folch et al (1957). After separation the aqueous phase was lyophilised and redissolved to a final volume of 1 to 5 ml in distilled water, to which an equal volume of absolute ethanol was added. The sample was allowed to stand at 4^{o}C for 17 to 18 hours, the precipitate removed by filtration, and the filtrate dried under a stream of dry nitrogen at 60^{o}C.

Derivatisation of samples for gas-liquid chromatography of sugars. The derivatisation method was that of Varma et al (1973a,b). The sample was redissolved in 0.6 ml of 2.5% (w/v) hydroxylamine in pyridine, sealed and incubated at 90^{o}C for 30 minutes. After cooling 1.8 ml of acetic acid was added, the sample vial sealed and again incubated at 90^{o}C for 30 minutes. The derivatised sample was dried under a stream of oxygen-free nitrogen in a sand bath at 40^{o}C and redissolved in chloroform.

Gas-liquid chromatography of sugars. The sample was analysed on a fused silica capillary column, 30 m x 0.316 mm, with a liquid phase of DB-5, film thickness of 0.25 um (part # 1235032, J and W

Scientific, 91 Blue Ravine Rd, Folsom, California). A flame ionization detector was used. The injector was a split/splitless system and a split ratio of 10:1 was used. Injector and detector temperatures were 260°C and 325°C respectively. The oven was temperature-programmed from 100 to 300°C at 4°C per minute, with a 10 minute hold at 300°C. The carrier gas (helium) flow was 2 ml/minute, the makeup gas (nitrogen) 28 ml/minute, air 300 ml/minute, and hydrogen 30 ml/minute. An internal standard, 1 ml glucoheptose, 0.25 mg/ml, was added to most samples. Addition of the standard before hydrolysis caused destruction of the standard. The standard was therefore added after the lipid extraction, when the lyophilised sample was redissolved in water prior to the addition of ethanol.

Chemicals.

Lithium dodecyl sulphate (LDS), horseradish peroxidase (HRP) Type IV-A, Tween 20 (Polyoxyethylene-sorbitan monolaurate), cholesterol, dolichol, silica gel type GF (particle size 10 - 40 M), Trizma base, Amido Back and Coomassie Brilliant Blue R-250 were obtained from Sigma Chemical Co.,St. Louis, MO. Pronase, also obtained from Sigma, was self-digested in 10 mg/ml solution for 3 hours at 37°C before use. 2-mercaptoethanol was obtained from Reidel-de Haen.
PVDF membrane (Immobilon™), pore size 0.45 um, was obtained from Millipore Corporation, Bedford, MA. 4-chloro-1-naphthol was obtained from Bio-rad, Oakland, CA. Hydrogen peroxide, AnalaR 60%, was obtained from BDH, Poole, England. Concanavalin A was supplied by Vector Laboratories Inc., Burlingame, CA. Gelatin was obtained from Becton, Dickinson and Co. All chemicals used for LDS-PAGE were obtained from Bio-Rad, Richmond, CA., except for sucrose and LDS. Chemicals used for silver staining of gels were those of the Bio-Rad silver stain kit (Cat. No. 161-0443), except for trichloroacetic acid and silver nitrate. Restriction enzymes and REact™1 reaction buffer were obtained from Bethesda Research Laboratories. 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 megaohms/cm. All other reagents were analytical grade and all solvents were double distilled.

CHAPTER 4

SUBCLINICAL PATHOLOGY AND LIPOFUSCIN ACCUMULATION IN THE EQUINE THYROID GLAND.

INTRODUCTION

Lipofuscin accumulation is reported to progressively increase with age in most organs (Munnell and Getty 1968, Porta and Hartroft 1969, Ambani et al 1977, Feeney-Burns et al 1987), although this is not always the case in the human thyroid (Matsubara et al 1982, Ohaki et al 1986). If the quantity of lipofuscin is primarily related to lysosomal activity rather than to age, the level of thyroid activity may affect the total quantity accumulating. In this chapter lipofuscin accumulation in equine thyrocytes is measured and compared to age, indicators of thyroid activity and histological abnormalities in the thyroglobulin stored extracellularly as colloid. Other thyroid changes, some of which have been previously reported and described as age-related, are also measured.

SPECIAL METHODS

Diameters of 30 follicles were measured on a linear traverse of the standard H&E stained paraffin section from each horse. In addition, each follicle was scored for the degree of peripheral scalloping of the colloid.

RESULTS

Thyroid size and colour

All thyroids used in this study were from horses free of obvious endocrine or systemic disease. Thyroids from the 31 horses of different ages were normal in gross appearance but varied considerably in weight, from 10 to 73 g thyroid tissue per horse (mean 30 g, s.d. +/- 14). The variation in weight was not age-related.

The thyroids from horses in their first decade of life was usually dark pink. In older horses they were tan or dark red-brown (Fig. 4:1). Age could not be predicted from the degree of browning.

Follicle size and activity

The follicle diameters and levels of macropinocytosis were measured in H&E stained paraffin sections. The average diameter of follicles was 180 um. Occasional small follicles containing no colloid were most likely to be tangential sections and were excluded from the study. No correlation between average follicle diameter (Table 4:1) and age, or the range of follicle diameters (Table 4:1) and age was discovered by linear regression. The percentage of the variance in follicle diameter due to the regression (the R^2 value) was only 0.50% of the total variance, while that of follicle diameter range was only 4.42%. In all sections, the most peripheral follicles, in a layer three to five follicles deep, had diameters roughly half those of the more central ones. All the thyroids showed macropinocytosis (Fig. 4:2) in at least some follicles, except that of the premature foal. No change in overall macropinocytotic activity with age was detected by linear regression of the average level of macropinocytosis onto age (Table 4:1). The percentage of the variance due to the regression (the R^2 value) was only 4.85% of the total variance.

Follicles with squamous thyrocytes and no evidence of macropinocytosis, the characteristics of 'cold' follicles (see Chapter 2), were rare or absent in most individuals regardless of age (Table 4:2). The few present were not unusually large compared to active follicles.

Lipofuscin

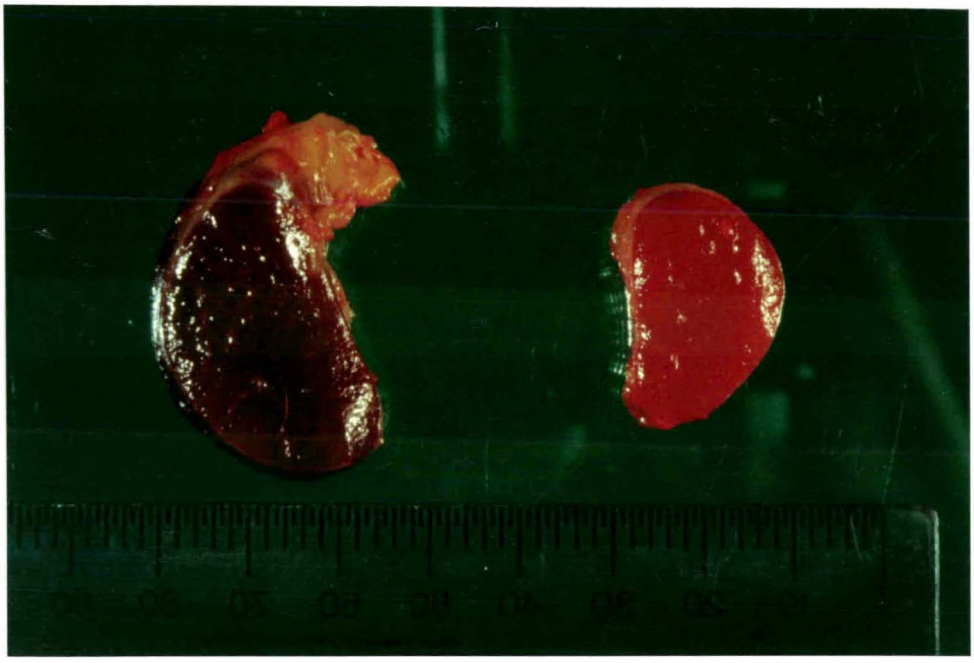
Lipofuscin granules were yellow-brown or brown in H&E stained sections. They were observed in the apical cytoplasm of the majority of thyrocytes in horses over 7 years of age (Fig. 4:2). The depth of colour of the granules was consistent within a section but did not correlate with age. In the majority of horses the number and size of

Figure 4:1

Gross appearance of fresh thyroid glands: Comparison of the cut surfaces of the thyroid glands of an 18 month old filly (left) and a 25 year old gelding (right) shows the gross colour change due to lipofuscin accumulation.

Figure 4:2

Histology of equine thyroid tissue: In cross section, follicles are composed of a central extracellular pool of thyroglobulin called the 'colloid' (T) surrounded by a single layer of thyrocytes. Thyrocyte cell nuclei (large arrowhead) stain purple or dark blue with this stain . Yellow-brown lipofuscin granules (small arrowhead) are present in the apical cytoplasm of thyrocytes. Fibrous connective tissue and capillaries are present between follicles. Most follicles in this figure show a large amount of scalloping of the colloid indicating a high level of macropinocytosis. The follicle at upper left is an exception. H&E x 100.



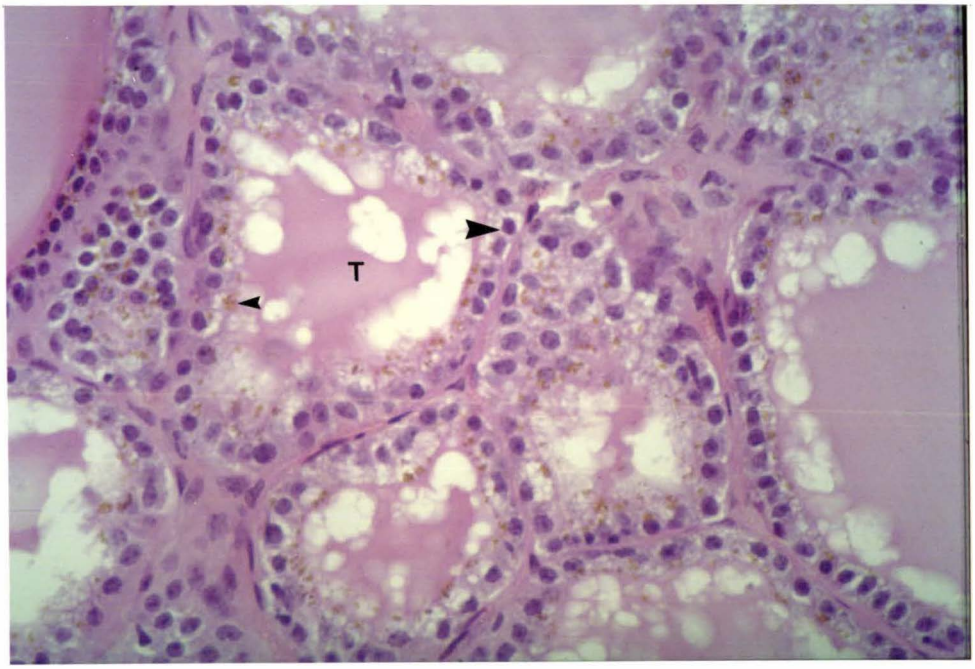

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Table 4:1. Thyroid activity, presence of colloid abnormalities and numbers of lipofuscin granules counted in plane of section

Horse	Mean follicle diameter (um)	Macropinocytosis* Mean	Range	Colloid Abnormalities (%follicles**)	Granules /50 cells	R^{2***}
PR	113 +/- 52	0	0	0	0	n/a
8M	215 +/- 72	9	0-20	7	n/a****	n/a
1A	160 +/- 59	11	0-15	5	0	n/a
3A	249 +/- 126	2	0- 5	5	15	30%
3B	151 +/- 91	14	0-20	0	23	22%
5A	160 +/- 109	12	0-20	16	56	55%
6A	229 +/- 116	5	0-10	7	50	59%
6B	195 +/- 77	13	5-20	5	45	59%
7A	148 +/- 64	15	0-20	6	29	71%
9A	171 +/- 86	14	5-20	5	62	59%
12A	249 +/- 111	1	0-10	3	35	61%
12B	118 +/- 233	8	0-20	2	30	61%
12C	195 +/- 111	14	0-20	8	94	39%
15A	225 +/- 111	5	0-20	14	50	75%
15B	262 +/- 105	15	0-20	18	28	78%
15C	221 +/- 105	15	0-20	13	87	79%
20A	93 +/- 32	20	0	2	54	94%
20B	193 +/- 94	14	0-20	8	69	76%
20C	117 +/- 38	12	0-20	35	82	75%
20D	116 +/- 48	20	0	2	86	74%
20E	214 +/- 123	4	0-20	15	61	57%
20F	148 +/- 133	13	0-20	9	44	77%
25A	204 +/- 165	9	0-20	22	92	88%
25B	199 +/- 86	19	0-20	47	45	78%
25C	206 +/- 133	10	0-20	36	97	52%
25D	254 +/- 128	14	0-20	46	76	46%
25G	254 +/- 143	7	0-20	28	40	84%
28A	97 +/- 76	10	0-20	32	87	76%
28B	195 +/- 123	10	0-20	21	36	82%
30A	187 +/- 83	14	0-20	31	40	52%
35A	209 +/- 128	20	10-20	6	41	84%

*Key to macropinocytosis:

0 = None. 5 = Light, < 50% of colloid diameter.

10 = Light >50% of colloid diameter. 20 = Heavy, >50% of colloid diameter.

** Some follicles contained more than one type of colloid abnormality.

*** The percentage of the variance in granule numbers due to the variance in nuclei numbers.

**** n/a = not applicable; 8M was not suitable for this statistical analysis as only 5% of follicles in the section contained lipofuscin.

Table 4:2 Presence of cold follicles

Horse	Cold follicles
PR	nil
8M	nil
1A	nil
3A	nil
3B	nil
5A	1-2 per x4 field
6A	1-5 per section
6B	nil
7A	nil
9B	nil
12A	nil
12B	nil
12C	1-5 per section
15A	1-5 per section
15B	1-2 per x4 field
15C	1-2 per x4 field
20A	1-2 per x4 field
20B	nil
20C	nil
20D	nil
20E	1-2 per x4 field
20F	1-5 per section
25A	3-10 per x4 field
25B	1-5 per section
25C	nil
25D	1-5 per section
25G	nil
28A	3-10 per x4 field
28B	nil
30A	nil
35A	nil

lipofuscin granules per cell, as seen in plane of section, appeared even throughout the gland. However, in horses younger than 7 years, lipofuscin granules were particularly large in thyrocytes of the small proportion of follicles containing abnormal colloid, compared to those in the normal follicles. There was no age-related increase in granule size. Some horses in their twenties had particularly small granules, and the largest granules were found in abnormal follicles in the 5 year old horse. Small numbers of lipofuscin-laden macrophages were observed in the interstitial connective tissue between follicles in several horses over 20 years of age.

Changes in colloid

In most follicles the colloid stained an even pink colour with H&E, although knife chatter (Fig. 4:3,Fig. 4:4) was a frequent artifact of processing, due to the variable consistency of the tissue. A number of abnormalities in the appearance of the colloid were noted, some of which increased in incidence with age. Observed abnormalities were:

[a] **Zones of basophilia,** usually in a central position in the follicle and sharply demarcated from the normal colloid (Fig. 4:3). These tended to be more severely marked by knife chatter than normal colloid, suggesting greater hardness. In unstained sections the basophilic zones exhibited moderate yellow fluorescence (Fig. 4:5, Fig. 4:6), in contrast to the rest of the colloid.

[b] **Solid colloid fragments** with sharp edges and angles (Fig. 4:4). These were unlikely to be artifacts of processing, because fissures between fragments frequently contained cellular debris (Fig. 4:4). In contrast to normal colloid, this colloid exhibited yellow-green fluorescence in unstained sections.

[c] **Sparse shreds of colloid** in an otherwise empty follicle (Fig. 4:7). Colloid with this appearance was not fluorescent.

[d] **Nucleated cells,** presumed to be detached thyrocytes in various stages of autolysis (Fig. 4:4). These were fluorescent in unstained sections.

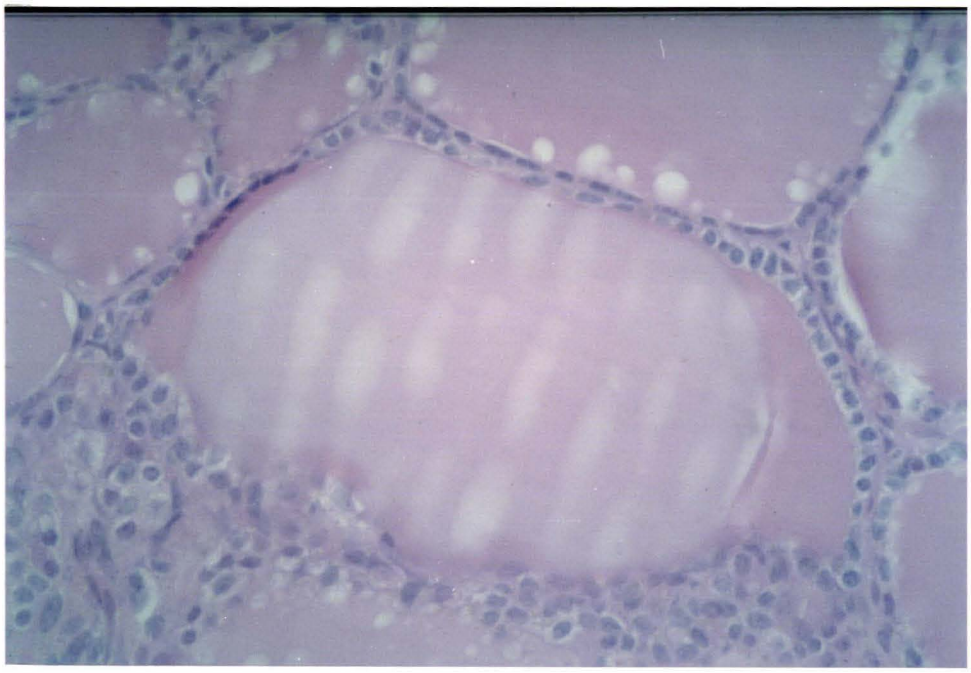
[f] **Spherites,** rounded basophilic structures, sometimes with an intensely basophilic centre which gave them the appearance of 'fried eggs' (Fig. 4:8). Spherites were often multiple in follicle lumina. In some cases they strongly resembled swollen autolysing cells, suggesting that at least some spherites represented a stage in thyrocyte autolysis. Spherites were intensely fluorescent (Fig. 4:5). In some, the central core was more fluorescent than the peripheral area (Fig. 4:5). The majority of spherites did not stain for calcium by von Kossa's method.

Figure 4:3

Basophilic zone in the colloid: The basophilic zone has a sharp border. It is marked by knife chatter, an artifact of sectioning. H&E x 100.

Figure 4:4

Solid colloid, divided into fragments: The colloid in the follicle at centre appears solidified and divided into fragments with sharply defined edges and angles. Cell nuclei and lipofuscin granules are present in the colloid of this follicle. The cell nuclei may be those of detached, autolysing thyrocytes. A number of follicles in this section, for example those at top centre and top right, show knife chatter, an artifact of sectioning. H&E x 40.



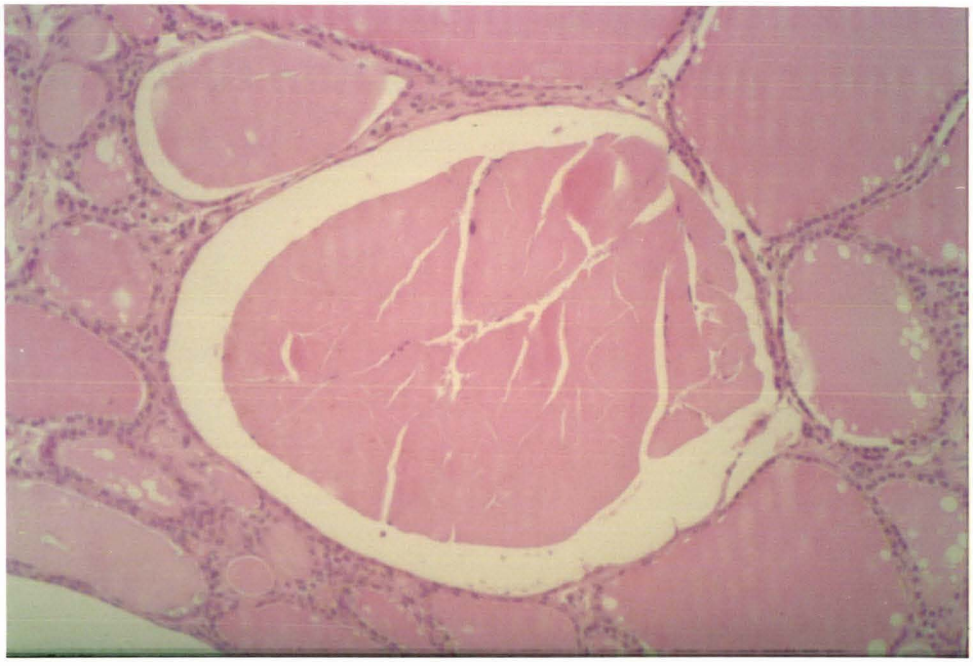
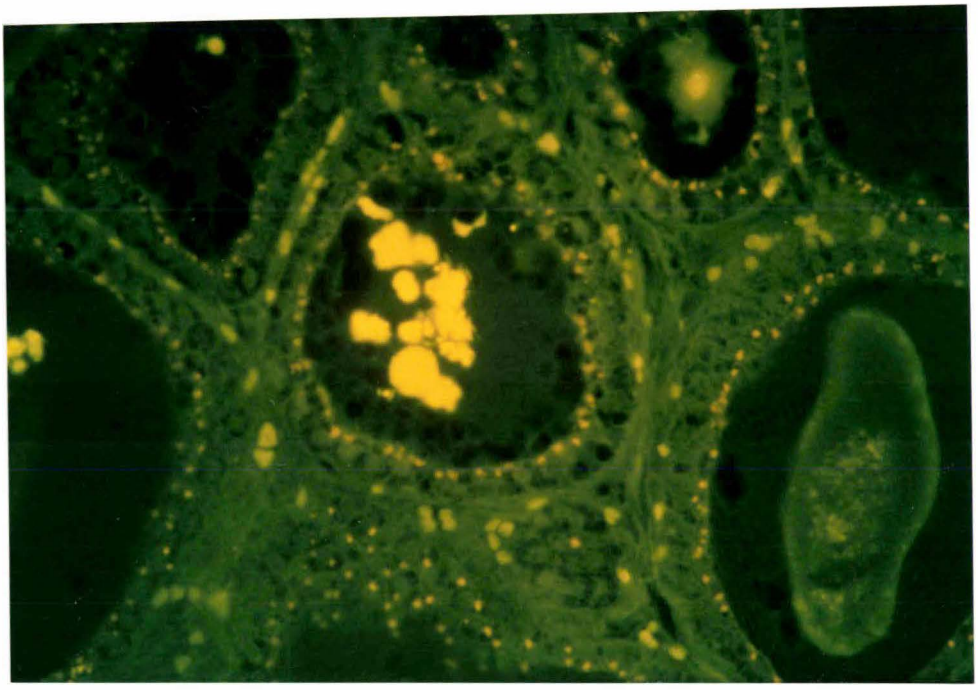


Figure 4:5

Fluorescence microscopy: The follicle at centre contains intensely fluorescent spherites. Lipofuscin granules in the apical cytoplasm of thyrocytes are intensely fluorescent. The large follicle at left also contains spherites. The small follicle at upper right contains a 'fried egg'-type spherite with greatest fluorescence at centre. The large follicle at lower right contains a moderately fluorescent area which in H&E section is a basophilic zone. Unstained x 400.

Figure 4:6

Fluorescence microscopy: The follicle on the left contains macrophages laden with intensely fluorescent lipofuscin. The follicle on the right contains a basophilic zone with lipofuscin granules in its periphery. Thyrocytes of all follicles contain intensely fluorescent lipofuscin. Unstained x 400.



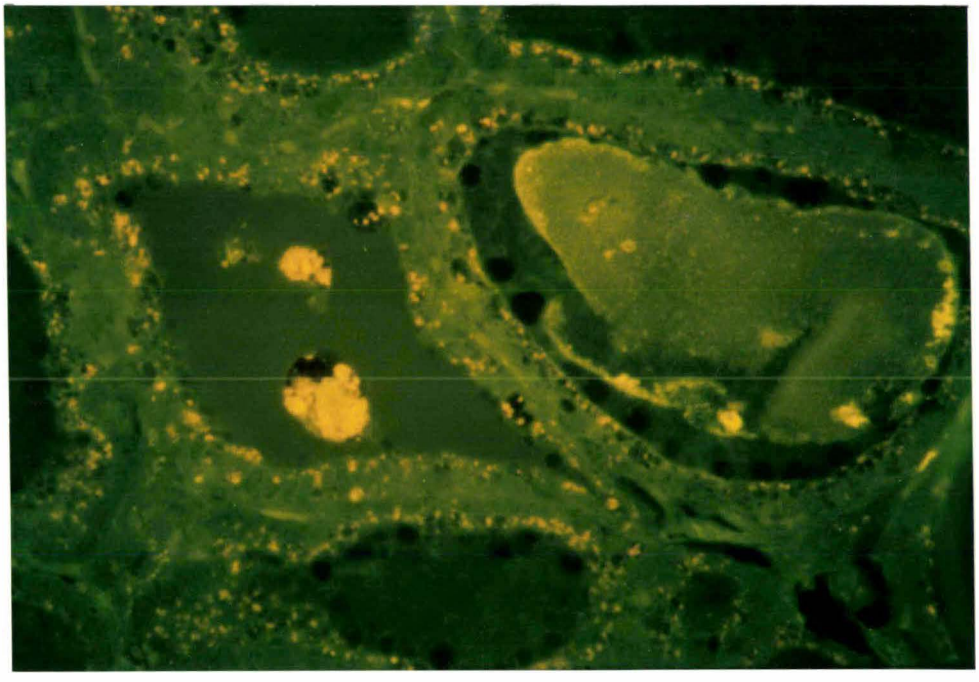
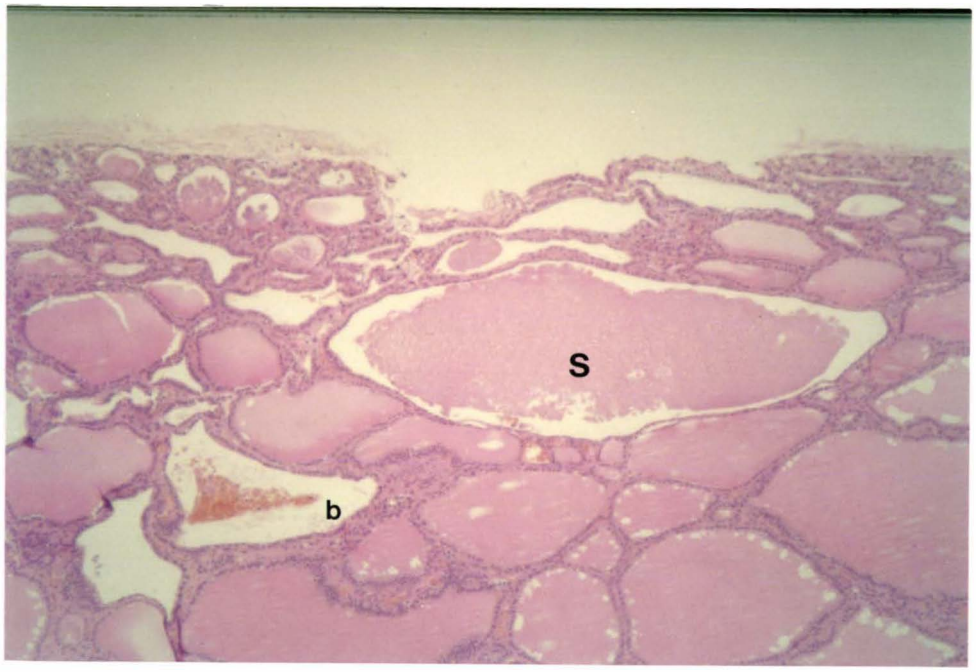


Figure 4:7

Shreds of colloid and erythrocytes in follicles: One follicle contains shreds of colloid (S) while another contains erythrocytes (b) and a small quantity of shreds of colloid. The follicles immediately beneath the gland capsule (at top) are smaller than those deeper within the gland. H&E x 40.

Figure 4:8

Spherites: 'Fried egg'-type spherites in the colloid of the follicle at centre are cell-like in appearance and may develop from autolysing cells. H&E x 400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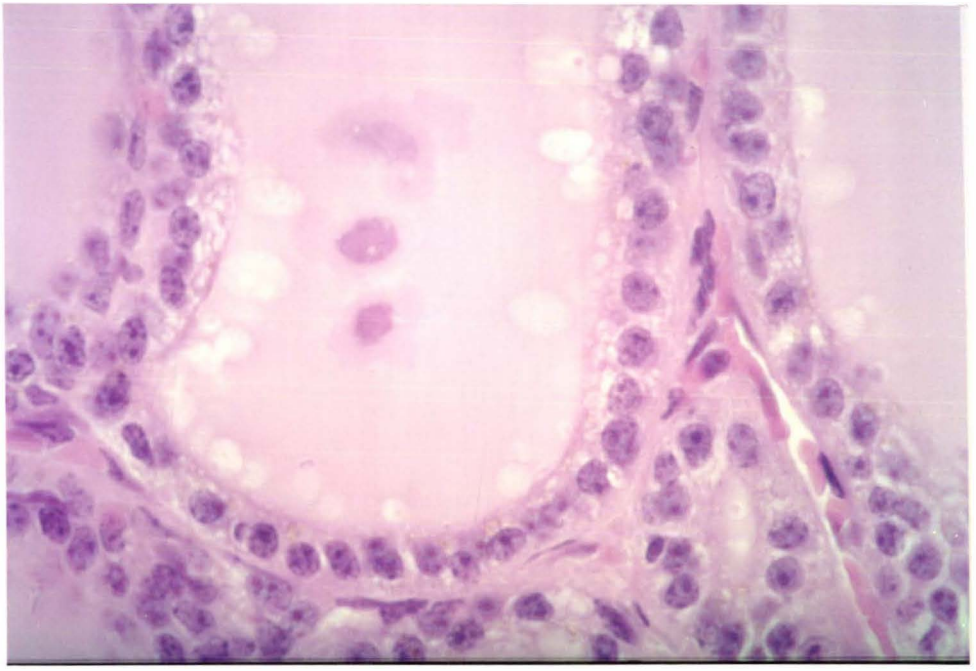
b



[g] **Lipofuscin granules**, which were observed free in the colloid (Fig. 4:9, Fig. 4:10), within detached thyrocytes, within spherites, and within basophilic zones (Fig. 4:10). They were also occasionally found as large clusters within macrophages in follicle lumina (Fig. 4:9). Such clusters were highly fluorescent (Fig. 4:6). Lipofuscin granules in the colloid were a rare observation in almost all horses (Table 4:3), and did not represent a major proportion of the total lipofuscin observed in the gland .

[h] **Calcium oxalate crystals**, which were irregularly shaped and colourless in H&E stained sections (Fig. 4:9), birefringent (Fig. 4:10) and showed a glassy grey to black reaction to von Kossa's stain for calcium.

[i] **Erythrocytes**, apparently normal or slightly pale (Fig. 4:7).

In each H&E section, 20 high power (x40 objective) fields were examined sequentially across the widest diameter of the section. The number of follicles in each field was recorded, as were the numbers of follicles containing abnormalities of colloid (Table 4:1). Follicles containing abnormal colloid were rare in almost all individuals under 15 years of age. They increased slightly in prevalence from 15 to 25 years of age. In contrast the percentage of follicles containing abnormal colloid was considerably higher in the majority of horses 25 years and over than it was in younger horses (Table 4:1). Although age-related, the trend was not linear, and some aged horses, including the oldest horse in the study, had few such follicles.

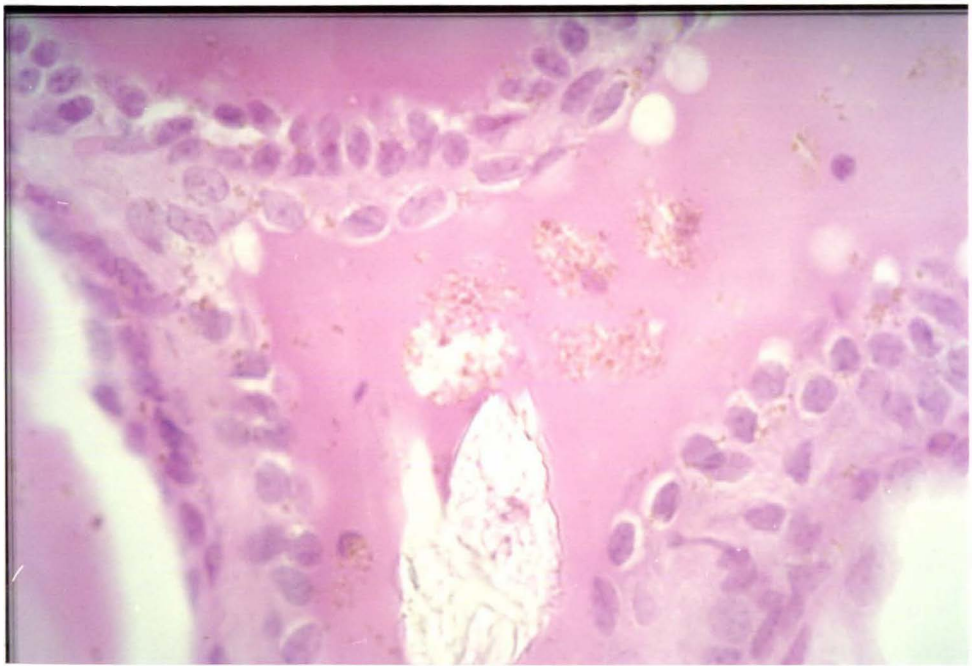
The percentage of follicles showing each type of change are shown in Table 4:3. The sum of the percentages shown for each horse in this table does not always equate to the figure in Table 4.1, as some follicles contained more than one type of abnormality. There was a trend for the prevalence of spherites, lipofuscin in the colloid, nucleated cells in the colloid, and shreds of colloid to increase with age. In contrast there was no relationship between age and the prevalence of calcium oxalate crystals, erythrocytes, basophilic zones, and solid fragments of colloid.

Figure 4:9

Multiple abnormalities in the colloid: The colloid of this follicle contains a calcium oxalate crystal, pigment-laden macrophages, extracellular lipofuscin and an isolated nucleus which may be the pyknotic nucleus of an thyrocyte undergoing autolysis. H&E x 400.

Figure 4:10

Multiple abnormalities in the colloid: The central follicle contains a basophilic zone in which lipofuscin granules and a birefringent calcium oxalate crystal are present. The thyrocytes of this follicle are squamous, and the lipofuscin granules in them are located lateral to the cell nuclei rather than the usual apical location. H&E x 100 with polarising filters. This photomicrograph was taken with an automatic camera which selected a long exposure time to compensate for the exclusion of light by the polarising filters. As a result the calcium oxalate crystal is overexposed and the background, which appeared dark grey to black to the eye, appears normally exposed.



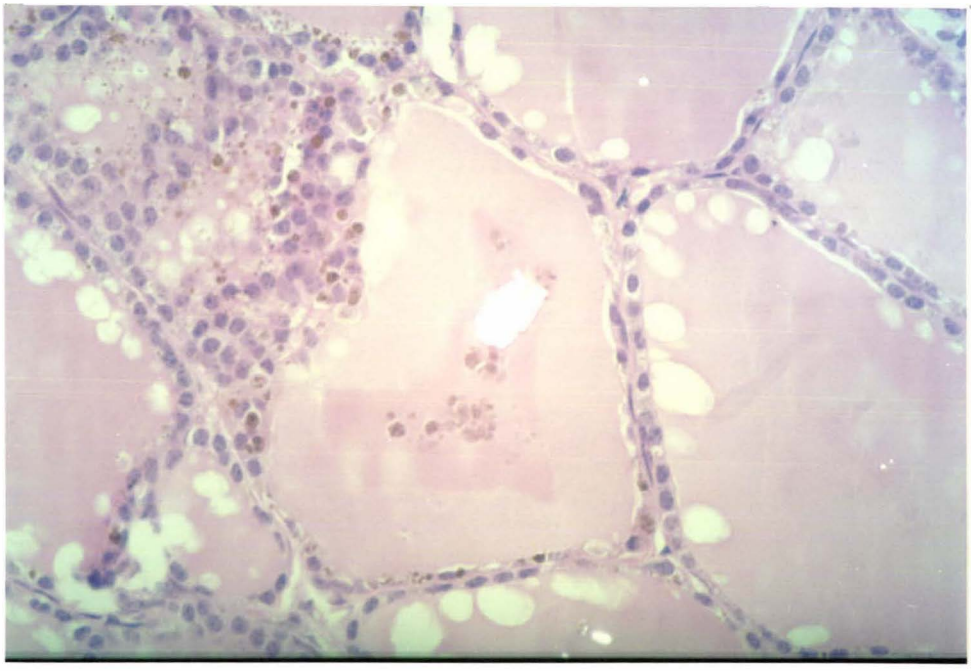


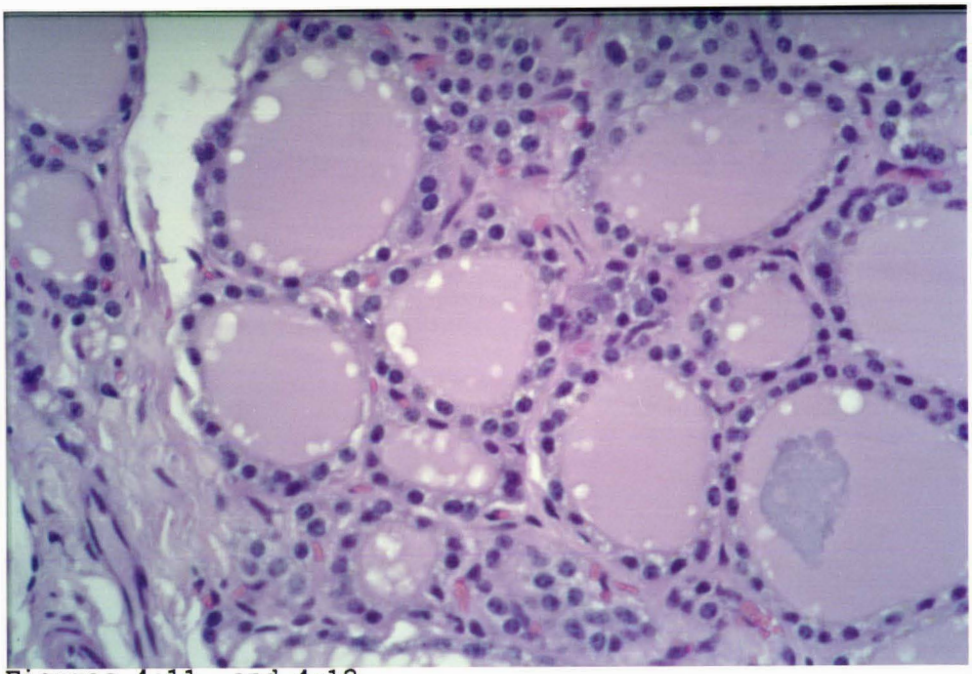
Table 4:3 Frequencies of types of colloid abnormalities observed

Horse	Nucleated cells	Lipofuscin	Erythrocytes	Basophilic zones	Oxalate crystals	Spherites	Fragments	Shreds
PR	-	-	-	-	-	-	-	-
8M	1	-	-	1	-	3	-	1
1A	-	-	-	2	-	2	-	-
3A	-	-	-	-	-	-	-	-
3B	-	-	-	-	-	-	-	-
5A	2	-	-	5	-	13	-	2
6B	-	1	-	2	3	1	-	-
7A	-	-	-	2	-	3	-	2
9A	2	-	-	-	1	2	-	-
12A	3	1	-	-	-	-	-	-
12B	1	-	-	-	-	-	-	1
12C	1	3	2	1	1	2	-	1
15A	3	-	-	1	-	7	3	5
15B	3	-	1	2	1	12	1	3
15C	2	-	-	7	1	4	1	1
20A	-	-	-	-	-	1	-	1
20B	1	-	-	1	1	6	-	-
20C	-	1	-	2	1	32	-	-
20D	-	-	-	-	-	-	-	1
20E	3	-	-	-	1	-	5	2
20F	2	-	-	-	-	5	-	2
25A	2	2	-	4	1	10	4	-
25B	10	1	10	3	3	25	1	23
25C	8	8	-	1	4	24	-	-
25D	3	12	2	-	-	36	-	1
25G	6	3	2	1	5	13	-	7
28A	3	8	-	3	4	25	-	-
28B	9	1	1	1	1	11	-	6
30A	10	2	1	-	-	18	2	1
35A	-	-	1	-	1	3	-	2

Some changes tended to occur together in the same follicle, but others did not. In 83% of follicles in which spherites were observed, no other change was present. The same was true of 79% of follicles containing basophilic zones, 68% of follicles containing fragmented colloid, 63% of follicles containing shreds of colloid, and 77% of follicles containing calcium oxalate crystals. In contrast, lipofuscin in the colloid was accompanied by other abnormalities in 78% of occurrences. In 59% of follicles in which lipofuscin was present in the colloid, spherites were also present, in 12% nucleated cells were present, and in 6% spherites and nucleated cells were present together. Occasionally lipofuscin granules were embedded in spherites. In 62% of follicles in which erythrocytes were present in the colloid, other abnormalities were also present. These included nucleated cells and shreds of colloid (28%), nucleated cells, shreds of colloid and spherites (17%), and nucleated cells and lipofuscin (7%). The presence of nucleated cells was also usually accompanied by one or more of the other abnormalities.

Thyrocyte lipofuscin related to colloid abnormalities

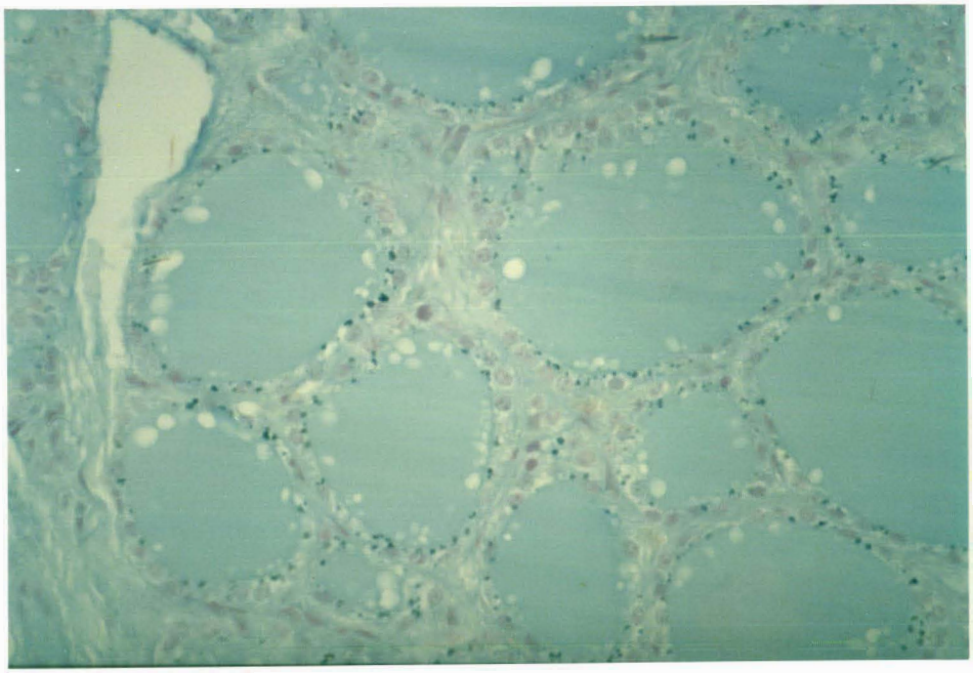
Colloid abnormalities were most obvious in sections stained with H&E, but lipofuscin was most obvious in sections stained with Schmorl's stain (Fig. 4:11, Fig. 4:12). Comparison of the different stains on serial sections was necessary to determine the relationship between lipofuscin in thyrocytes, age, and colloid abnormalities. Lipofuscin granules in follicles of horses up to 7 years of age were particularly large in the thyrocytes of follicles exhibiting colloid abnormalities. In the 8 month old foal and the 1 year old foal, lipofuscin was found only in the thyrocytes of a small number of follicles which contained basophilic, often fragmented, colloid. No granules could be found in normal follicles in unstained, H&E-stained or Schmorl's-stained serial sections from these two foals. Spherites or basophilic zones were present in 5% of follicles in the 3 year old horse 3A, and 15% of follicles in the 5 year old horse. The thyrocytes of these abnormal follicles contained large, intensely coloured lipofuscin granules (Fig. 4:13). No granules could be seen in the thyrocytes of normal follicles in the H&E stained section from



Figures 4:11 and 4:12.

Comparison of H&E and Schmorl's stain: Serial sections from the thyroid gland of 5A stained with H&E (above) and Schmorl's stain (below). Lipofuscin is much more readily found using Schmorl's stain, while the basophilic zone in the colloid (lower right) is more obvious using H&E. In the Schmorl's stained section, the dark blue lipofuscin granules are clearly contrasted with the pale blue staining of colloid and cytoplasm and the pink counterstaining of the cell nuclei.

H&E x 100. Schmorl's x 100.



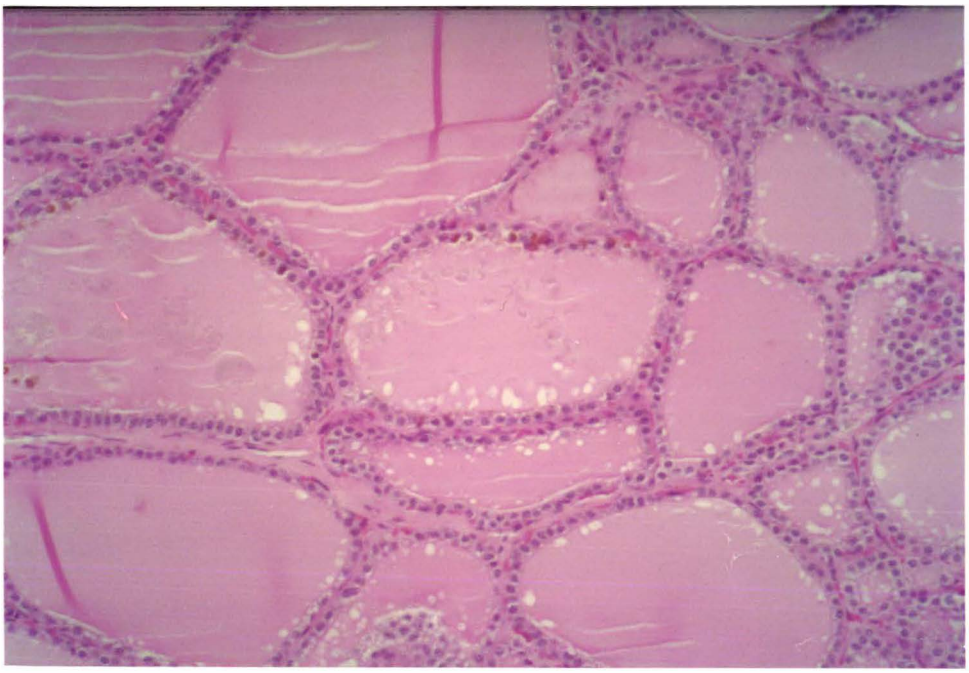


Figure 4:13.

H&E-stained section of the 5 year old horse: Large lipofuscin granules are present in the thyrocytes of the two follicles (centre and centre left) in which the colloid contains spherites. Macropinocytosis is occurring in these abnormal follicles. The abnormal follicle at centre left contains lipofuscin granules which are free in the colloid. Using this stain, lipofuscin granules are not apparent in the thyrocytes of the follicles in which the colloid is normal. The knife chatter marks in the colloid of most follicles, and the folds in the colloid of the follicles at upper centre and lower left, are artifacts of sectioning. H&E x 40.

these two horses (Fig. 4:13), but on examination of unstained serial sections by light microscopy, small yellow granules were observed. These granules stained with Schmorl's stain in serial sections. Similar granules were found in the follicles of 3B, a horse in which no colloid abnormalities or associated large granules were found. Lipofuscin granules of the small number of follicles containing abnormal colloid were also larger and more intensely coloured than those of normal follicles in the 6 year old horses, in which lipofuscin granules were also observed in normal follicles in H&E stained sections. The abnormal follicles with large lipofuscin granules were particularly obvious but affected only a small number of follicles in each horse and therefore did not appear to contribute greatly to the overall burden of lipofuscin in the tissue, which remained relatively low in the young horses (Table 4:1). Colloid abnormalities became more common in older horses, but they were not similarly accompanied by particularly large lipofuscin granules. The colour, size, and number per cell of lipofuscin granules showed little variation within sections from older horses.

Although the particularly large granules were found only in association with abnormal colloid in H&E sections from the 8 month old foal, the 1 year old foal, the 3 year old horse 3A and the 5 year old horse, follicles were found in H&E stained sections from all these horses which contained abnormal colloid but in which the thyrocytes did not contain unusually large lipofuscin granules.

Lipofuscin granule numbers per cell in section

Choice of sampling site: A sampling site 5 to 10 mm from a pole of a gland had the advantage that the whole of the section could be fitted onto the slide. To ensure that the quantity of lipofuscin at this site was representative of both glands, a preliminary study was made using the thyroids of two horses, 20B and 35A. Sections were cut from the site close to a pole of a gland, from the widest circumference of the same gland, and from the widest circumference of the other gland of the pair. Counts were performed on 30 follicles in each section and linear regressions calculated. In the case of each horse, the three lines fell within the standard deviations of

each other, indicating that the peripolar sampling site was representative of both glands.

Choice of stain: Lipofuscin granules appeared more obvious when stained than when recognized by their natural colour in H&E sections. Therefore, serial sections of the thyroid of 25G, stained with PAS, Schmorl's and H&E stains, were compared. The number of granules and the number of cells in plane of section were counted under the x40 objective in 30 follicles in each section. Linear regression of the number of lipofuscin granules onto the number of cell nuclei gave linear equations which were solved for 100 cells (Table 4:4).

Table 4:4 Lipofuscin granules per 100 cells in differently stained sections from horse 25G

Stain	Lipofuscin	R^{2*}
H&E	49/100 cells	78%
PAS	73/100 cells	86%
Schmorl's	78/100 cells	85%

*R^2, the coefficient of determination, indicates the percentage of the variability in Y (granule number) due to the variability in X (nucleus number). The high R^2 values indicate that in all cases the correlation between the number of cell nuclei and number of granules was high.

Lipofuscin granules were most readily counted in sections stained with Schmorl's stain, because this stain gave the greatest contrast between the granules and other structures in the section, and therefore the least risk of confusion between granules and other structures. Granules in the normal follicles of the young horses between 3 and 7 years of age were stained with Schmorl's stain but could not be seen in the H&E stained section (Fig.s 4:11 and 4:12). PAS was comparable to Schmorl's as a stain for lipofuscin (Table 4:4), but other structures in the section, such as thyroglobulin, were also PAS-positive (Fig. 5:2), and this lack of colour discrimination made PAS inferior to Schmorl's. Of particular concern was the risk of mistaking colloid droplets in the cytoplasm of thyrocytes for lipofuscin granules.

Enumeration of granules and cells per follicle: Thirty follicles were selected from a Schmorl's-stained paraffin section from each horse, the only criteria for selection being:

i) That follicles were sectioned transversely so that individual cells were visible from basal membrane to apical membrane (Fig. 4:12) rather than tangentially (as in Fig. 5:1).

ii) That none of the thyrocytes were distorted or obscured by artifacts such as detachment of thyrocytes from the basement membrane, or folds in the section.

iii) That a range of follicle sizes was represented.

Linear regressions were calculated and a hypothetical follicle containing 50 cells inserted into the fitted equation (Table 4:1). Lipofuscin accumulated rapidly in the first few years of life but after 5 years the number of lipofuscin granules in the thyroid gland of the horse was not correlated to the age of the animal. Animals of the same age differed greatly in the number of lipofuscin granules per follicle.

The relationship between number of cells in a follicle and number of granules was linear in 24 of the 28 animals studied. This is illustrated by the high R^2 value obtained for most individuals (Table 4:1) which is a measure of the fit of the linear model. However, when data from all the horses were pooled (Fig. 4:14) the overall relationship was a curve. The relationship was nearly linear up to a follicle size of approximately 100 cells, after which the number of granules per cell declined. For this reason the number of granules was calculated for a follicle of 50 cells (Table 4:1). The great majority of follicles examined contained less than 100 cells in cross-section, so the curve was caused by a minority of the total number of follicles examined.

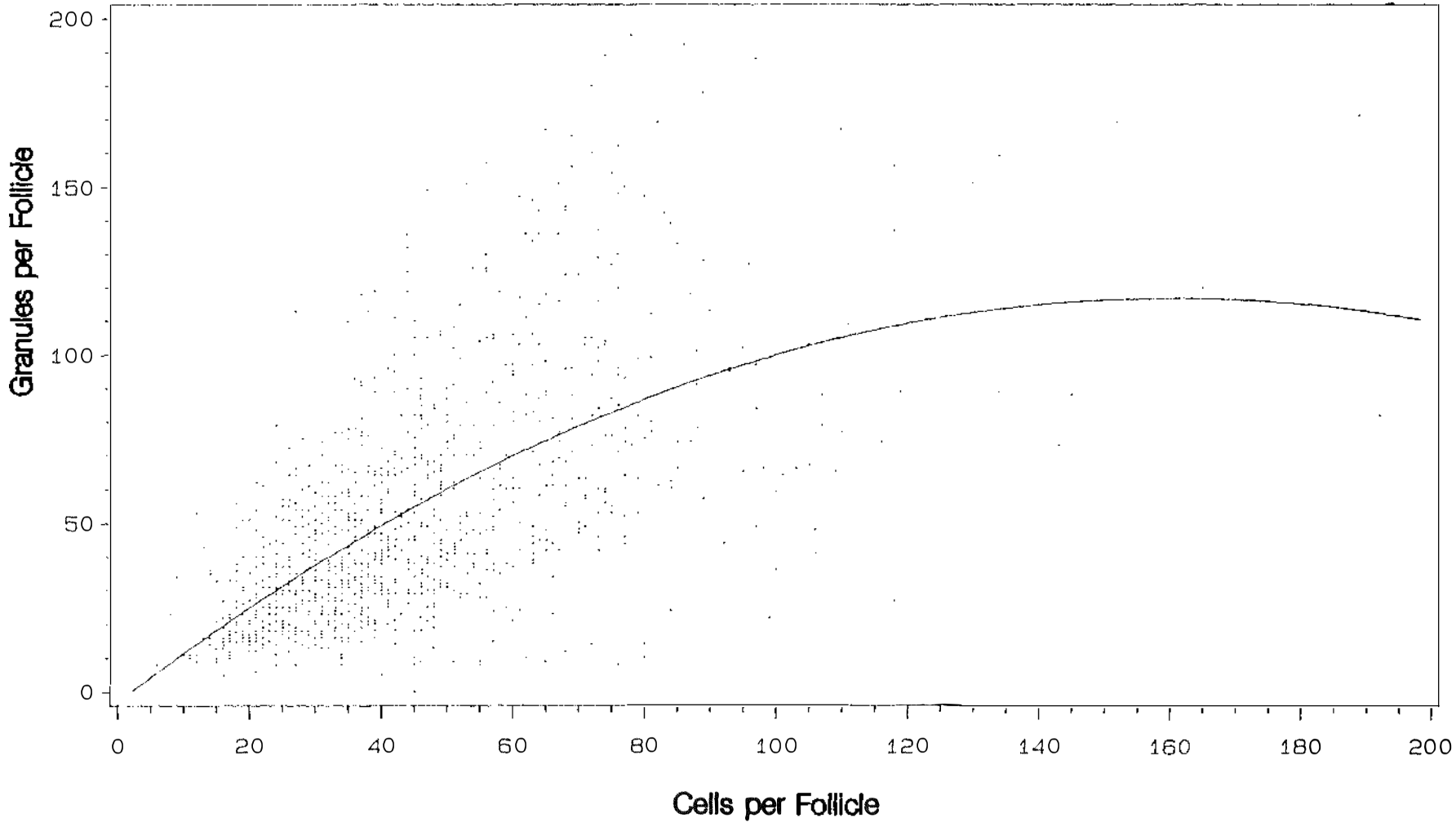
Lipofuscin and follicle activity

Thirty follicles in each section were scored for the level of peripheral scalloping of the colloid (Fig. 4:2), a histological

Figure 4:14

Lipofuscin granules per cell. Data in this graph are pooled from all horses included in the granule counting study. Each point on the graph represents one follicle in plane of section. Most follicles contain less than 100 cells in section, and the relationship between granule numbers and follicle size is approximately linear for these follicles. However among the small number of follicles exceeding 100 cells in section, there is a trend for fewer lipofuscin granules per follicle as cell numbers increase.

Granules Per Cell



indication of macropinocytosis and hence metabolic activity. An average score for the level of macropinocytosis in the gland was calculated (Table 4:1). The number of lipofuscin granules per 50 cells was compared to this score by linear regression. The percentage of the variance due to the regression (the R^2 value) was only 9.45% of the total variance, indicating that the two phenomena were not related. In two horses, 20A and 20D, all follicles showed heavy scalloping of the colloid. This heavy macropinocytosis was not associated with either an increased or decreased quantity of lipofuscin compared to the other horses in the large cohort of 20 year old horses (Table 4:1).

Lipofuscin and follicle size

The number of Schmorl's-positive lipofuscin granules per 50 cells was also compared to the mean and range of follicle diameters for each horse (Table 4:1). No correlation was found between average follicle size and lipofuscin accumulation (R^2 = 0.29%), or between the range of follicle sizes and lipofuscin accumulation (R^2 = 0.69%).

Lipofuscin and frequency of abnormal colloid

In light of the apparent association in young horses between particularly heavy lipofuscin accumulation and abnormalities in the colloid, lipofuscin granule numbers in Schmorl's stained sections were compared to the occurrence of altered colloid in H&E stained sections from the same horses (Table 4:1) by linear regression. No correlation was found between granule numbers and frequency of colloid abnormalities (R^2 = 17.41%). The subjective view that there was an association between granule numbers and frequency of colloid abnormalities in H&E sections from young horses was almost certainly due to the large size of the granules, which made them more obvious.

DISCUSSION

The quantity of lipofuscin increased between infancy and maturity. However after the first 5 years of life there was no correlation between the number of lipofuscin granules and age. Therefore the definition 'age pigment' was not appropriate to this lipofuscin. On

the other hand the term 'ceroid' was not appropriate either, because it was not usually associated with pathological change, and there was no evidence for the involvement of lipid peroxidation (see Chapter 1). Consequently the lipopigment is referred to as lipofuscin throughout this thesis. Intensity of granule colour did not increase with age in horses over 5 years, and there was no increase in size.

The results of this study are in contrast to the conclusions of Matsubara et al (1982) and Ohaki et al (1986), that there is a general increase in lipofuscin quantity with age in human thyroids. However, in both those studies some thyroids, even from individuals in their eighth or ninth decade, contained no lipofuscin.

Lipofuscin is generally assumed to be an inert end-product, resistant to both catabolism and excretion (Dolman and Macleod 1981). If this were true, then the quantity of lipofuscin in the thyroid should progressively increase with age. Since lipofuscin granules do not become more numerous or increase in size with age, it is likely that lipofuscin in this tissue can be catabolised, and therefore is an intermediate stage, rather than a terminal stage, of lysosomal catabolism. Some excretion of lipofuscin granules may also occur. Lipofuscin granules observed in the colloid could have been excreted from living thyrocytes, although, since they were usually found in abnormal colloid, it is more likely that they were released from exfoliated thyrocytes. Macrophages containing lipofuscin were also occasionally observed in the colloid. Such macrophages may migrate to become the lipofuscin-laden macrophages occasionally observed in the interstitial connective tissue, forming a pathway for gradual removal of lipofuscin. Alternatively, the macrophages in the interstitium may have been involved in the phagocytosis of thyrocytes of follicles which have undergone complete involution. It is possible that these macrophages catabolise the phagocytosed lipofuscin or export it from the gland. However these macrophages were only occasionally observed so it is unlikely that they represent a major pathway for the removal of lipofuscin from the thyroid gland.

Thyroid lipofuscin granules are lysosomal in nature (Ives et al 1975). Substrate may enter lysosomes by one of three processes;

autophagy, endocytosis (heterophagy), or crinophagy. Autophagy is the lysosomal degradation of constituents of the cell itself. Endocytosis is the degradation of material which originates outside the cell. Crinophagy is the lysosomal degradation of secretory material which is not secreted (de Duve 1969, Farquhar 1969).

The major source of material for lysosomal degradation in thyrocytes is endocytosis of colloid, and material found in lipofuscin is therefore most likely to originate from endocytosis. The low numbers of lipofuscin granules in exceptionally large follicles (Fig. 4:14) supports this hypothesis, because very large follicles are less active in endocytosis than smaller follicles (Tice 1977). The association of colloid abnormalities with increased lipofuscin formation observed in the young horses may, at least in part, reflect attempted catabolism of thyroglobulin that has undergone some physical or chemical change. Autophagy of cellular constituents probably also contributes to lipofuscin, although it would represent a minor proportion of substrate in normal thyrocytes.

True crinophagy, without prior export of the thyroglobulin to the colloid, has not been reported in thyrocytes. Unlike other secretory cells, the thyrocyte stores secretory product as inactive prohormone in an extracellular position. Both synthesis and endocytosis of thyroglobulin are stimulated by TSH (Tice 1977), but as thyrocytes become less active, endocytosis ceases before secretion of newly formed thyroglobulin ceases (Studer et al 1978, Gerber et al 1987) with the result that the colloid pool is large in follicles of low activity (Tice 1977, Studer et al 1978, Gerber et al 1987). Since thyrocytes can store excess supplies of thyroglobulin in this way, true crinophagy is not likely to be required to regulate it.

Eventual cessation of thyroglobulin secretion, after endocytosis has ceased, may be due to compression of the apical membrane and cytoskeleton by the excessive quantity of colloid in the follicle (Studer et al 1978, Gerber et al 1987). Theoretically, crinophagy might be necessary if thyroglobulin secretion was prevented in this way. If crinophagy were to occur under these circumstances and lead

to lipofuscin accumulation, then lipofuscin would be most abundant in large, inactive follicles. In this study, very large follicles with over 100 thyrocytes in cross-section characteristically contained less lipofuscin, rather than more, than smaller follicles (Fig. 4:14). Overall there was no correlation between the number of lipofuscin granules and follicle size or macropinocytotic activity.

A pathological failure of exocytosis could also lead to crinophagy. However, a failure of exocytosis would lead to a failure of endocytosis, because exocytosis of newly synthesised thyroglobulin always precedes macropinocytosis of colloid (van den Hove-Vandenbroucke 1980). The observation of macropinocytosis in a follicle is therefore evidence that exocytosis is functioning normally. Lipofuscin is found in thyrocytes in which macropinocytosis is occurring (Fig. 4:2, Fig. 4:12), which militates against lipofuscin being produced by crinophagy.

There is evidence that lipofuscin in other tissues accumulates more rapidly as cellular activity increases (see Chapter 1). Mean follicle size and mean macropinocytotic activity are indices of follicle activity. Macropinocytosis was a feature of all the equine thyroids except that of the premature foal, and was not accompanied by other signs of thyroid hyperactivity such as columnar thyrocytes or paucity of colloid. Therefore it appears that macropinocytosis is a normal feature in the equine thyroid, as it is in the thyroid of the rat (Lupulescu and Petrovici 1968). No correlation between the indices of follicular activity and the quantity of lipofuscin in the thyrocytes was found. However macropinocytosis is a transient phenomenon, which can change dramatically within 30 minutes (van den Hove-Vandenbroucke 1980), and follicular diameter can also change in a few days under experimental conditions (Mahmoud et al 1986), so neither can be regarded as indicators of the long-term status of the thyroid gland. The few follicles in which over 100 cells were counted contained less lipofuscin per cell than the majority of follicles (Fig. 4:14). The exceptional size of these follicles indicates that they are less active in endocytosis and catabolism than the smaller follicles (Tice 1977).

The abnormalities in the colloid, such as basophilic zones, solid colloid fragments, and spherites, are indicative of alteration in the physical state of the thyroglobulin. Spherites have the same morphology as the 'basophilic granules' described by Capen (1985) and the 'Psammoma bodies' of Berthezene and Greer (1974) who showed that these structures contain thyroglobulin. In this study some spherites had a 'fried egg'-like appearance which suggested a cell nucleus and cytoplasm. This morphology suggests that detached, autolysing thyrocytes may serve as foci for spherite formation, an argument supported by the association of lipofuscin and nucleated cells in the colloid of the same follicle as spherites. Furthermore, spherites were usually also present in the small number of follicles in which lipofuscin was observed in the colloid. If lipofuscin is released into the colloid from dying thyrocytes, the thyrocytes may be observed as nucleated cells early in autolysis and as spherites in advanced autolysis.

Pulmonary 'corpora amylacea' which are found in association with chronic nonprogressive pneumonia in lambs have been shown to contain deoxyribonucleoproteins, ribonucleoproteins, and glycoproteins, and corpora amylacea have also been reported in bovine mammary gland and human prostate (Lin et al 1989). Corpora amylacea resemble the spherites found in equine thyroid in that they are ovoid and concentrically laminated, and it appears probable that cellular constituents contribute to both.

While most types of colloid abnormality were usually the only change present in a follicle, lipofuscin in the colloid, nucleated cells in the colloid, and erythrocytes in the colloid were usually accompanied by one or more of the other abnormalities, and are likely to be consequences of them.

Lipofuscin accumulation was particularly heavy in the small proportion of follicles in young horses which contained abnormal colloid, and was restricted to such follicles in the 8 month old foal and the 1 year old foal. However it is unlikely that the development

of abnormalities in the colloid is a necessary precursor to lipofuscin formation in thyrocytes. Abnormalities in the colloid were rare in most individuals under 25 years of age (Table 4:1), but lipofuscin became widely and evenly distributed throughout normal follicles in sections from horses of 5 years and over. In 3A, the 5 year old horse and the 6 year old horses, lipofuscin was also present in normal follicles, although the granules were smaller and less obvious than those in abnormal follicles. Similar small granules were present in the normal follicles of horse 3B.

Given the ubiquitous occurrence of lipofuscin in normal thyroid follicles of mature horses, it is likely that in most thyrocytes, lipofuscin granules are normal intermediates of lysosomal catabolism. The variation in the quantity of lipofuscin accumulating between mature horses could reflect differences in their metabolic rates, particularly the rate of proteolysis.

The strong association observed in young horses between abnormal colloid and numerous large lipofuscin granules may reflect either the attempted catabolism of abnormal substrate, or an impaired lysosomal system in the cells of the affected follicles. Catabolism of colloid could be delayed if the colloid was partially dehydrated or contained cellular debris. Alternatively, the primary defect could be impairment of lysosomal catabolism, with large quantities of lipofuscin, an intermediate stage of catabolism, accumulating in the cells. Under such conditions a feedback mechanism could exist to prevent further endocytosis of colloid, with the result that the colloid in the follicle lumen is stored for abnormally long periods and consequently develops abnormalities. However, if lysosomal impairment precedes colloid abnormalities, some follicles should be present with large lipofuscin granules but without colloid abnormalities. Such follicles were not present in the young horses, although follicles with abnormal colloid but without lipofuscin were present. This could indicate that abnormal substrate is the primary defect in the follicles, but this is unlikely to be the full explanation because abnormal colloid does not appear to provoke heavy accumulation of large lipofuscin granules in older horses.

Alternatively, the abnormal colloid and the lipofuscin accumulation may occur together, both reflecting some abnormality of follicular function. Nothing is known about how long each phenomenon persists. Abnormal colloid may take longer to resolve than lipofuscin, and the follicles containing abnormal colloid but without large lipofuscin granules may therefore be in a stage of recovery. Alternatively, these follicles may be less severely affected, so that colloid turnover is delayed sufficiently to allow colloid abnormalities to develop, but catabolism proceeds at a pace sufficient to prevent the buildup of large lipofuscin granules.

The absence of the large lipofuscin granules in older horses is difficult to explain, because abnormalities in the colloid occur in older horses and, particularly in horses of 25 years and older, increase in frequency. It is known that individual follicles vary in their inherent responsiveness to TSH (Tice 1977, Studer et al 1989) and it is probable that they also vary in their responsiveness to other factors, including those that cause a decline in lysosomal activity. The abnormal follicles in the young horses may be those at one extreme of a range of levels of lysosomal activity, with a particularly low inherent level which becomes apparent as the foal matures and its metabolic rate declines. The absence of such follicles in mature horses could be because these 'low activity' follicles have involuted and disappeared, or because they have 'recovered' and operate within closer limits of homeostasis. Colloid abnormalities in older horses may occur in the presence of healthy cells with healthy lysosomal systems, which can cope with abnormalities in the substrate, so that large lipofuscin granules do not form.

The considerable variability in the weight of thyroid glands used in this study was not related to age and was not due to the development of cold follicles. The age-related increase in range of follicle sizes reported in other species (Doniach 1978, Wollman 1980) was not found in the equine thyroid. No decline in follicle size in advanced age occurred, in contrast to that observed in senescent humans (Doniach 1978). The reported age-dependent increase in follicles

containing calcium oxalate crystals in human thyroid (Doniach 1978, Reid et al 1987), was not observed in the horse thyroid (Table 4:3).

SUMMARY

There is no age-related increase in the quantity, size or colour of lipofuscin granules except between infancy and maturity. It is therefore probable that lipofuscin in this tissue is not an inert end-product, but can be catabolised.

Lipofuscin is ubiquitous and evenly distributed in thyrocytes of normal follicles in mature horses. Very large follicles, which are likely to be less active than those of average size, contain less lipofuscin per cell. It is concluded that lipofuscin is probably a normal intermediate stage in lysosomal catabolism in adult horses. It is most likely that lipofuscin in thyrocytes is principally derived from endocytosed material.

Thyroid follicles containing abnormal colloid are rare in young horses but there is a marked increase in frequency of these follicles in horses of 25 years and over. Some abnormalities appear to be alterations in the physical properties of the colloid while others may arise from the addition of exfoliated thyrocytes.

Extracellular lipofuscin granules were sometimes present in the colloid, usually accompanied by other abnormalities of colloid. The presence of lipofuscin in the colloid is therefore most likely to occur secondary to other abnormalities.

In horses under 7 years of age, lipofuscin granules were particularly large in rare follicles containing abnormal colloid. However, accumulation of smaller lipofuscin granules in thyrocytes of normal follicles occurred concurrently in horses of 3 years and over, and the relationship between abnormal colloid and large lipofuscin granules disappeared entirely in horses over 7 years of age. Therefore, the presence of abnormal colloid is not a necessary precursor to the accumulation of lipofuscin in thyrocytes.

The follicles containing abnormal colloid and unusually large lipofuscin granules in the young horses may represent one extreme of a range of rates of lysosomal catabolism. Such follicles were not present in older horses, and may either involute and disappear, or their lysosomal function may have altered to become closer to that of other follicles.

CHAPTER 5

LIPOFUSCIN IN THE EQUINE THYROID GLAND: HISTOCHEMICAL AND MORPHOLOGICAL HETEROGENEITY

INTRODUCTION

Reactions to a range of histological stains and the complex ultrastructure described in the literature suggest that lipofuscin is heterogeneous in composition. As recorded in the previous chapter, the quantity of lipofuscin in equine thyrocytes is not age-dependent in adult horses, and some abnormalities in the colloid are associated with particularly large lipofuscin granules in the thyrocytes of young horses. This suggests that lipofuscin formation in thyrocytes is not merely the accumulation of inert residual debris from a single type of cellular 'accident' as proposed by the lipid peroxidation theory, and that thyroid lipofuscin can be at least partially catabolised. If so, this could also give rise to chemical heterogeneity.

This chapter reports the histochemical heterogeneity and ultrastructural characteristics of equine thyroid lipofuscin, and the ultrastructural relationship of lipofuscin granules to organelles in the same cell.

RESULTS

Fluorescence and Histochemistry

Unstained Sections: Because of the natural yellow colour of lipofuscin granules they could be found in unstained sections by light microscopy. Although lipofuscin granules could not be detected in normal follicles in H&E-stained sections from the 3 year old horses and the 5 year old horse (see Chapter 4), the thyrocytes of these follicles did contain fine yellow granules when unstained sections were examined by light microscopy. The lipofuscin granules

exhibited a yellow-orange fluorescence and appeared more numerous by fluorescence microscopy than by light microscopy in H&E stained sections (Fig. 4:5, Fig. 4:6).

H&E stain: Lipofuscin ranged in colour from yellow to dark brown in H&E stained sections (Fig. 5:1). The depth of colour of the granules, although even within a section, varied between horses. This variation could not be attributed to staining conditions because it was apparent between sections that had been processed and stained together. Granules were usually located in the apical part of the cell (Fig. 4:2) although in squamous thyrocytes they were lateral to the nucleus (Fig. 4:10). Many granules contained round 'vacuoles' which were clear or pale yellow, in contrast to the darker colour of the encompassing granule matrix (Fig. 5:1). Lipofuscin granules were less fluorescent in H&E stained sections than in unstained sections.

PAS stain: Lipofuscin granules stained positively with the PAS method (Fig. 5:2) but there was considerable variability in the staining intensity between horses which was not correlated with age (Table 5:1) and which was apparent between sections that were processed and stained together. In most cases the native brown or yellow colour of the granules intruded. Because the 'vacuoles' did not stain with PAS, but appeared clear or pale yellow, granules were observed as crescent-shaped, PAS-positive areas of matrix partially surrounding 'vacuoles'. Lipofuscin granules were more obvious in PAS stained, than in H&E stained sections. With careful examination they could be distinguished from colloid droplets in the thyrocyte cytoplasm, because lipofuscin almost always retained some natural yellow colour. In contrast, colloid droplets stained more intensely with PAS, had no yellow tinge, and were oval or circular in shape.

Schmorl's stain: With Schmorl's stain, great contrast was obtained between the lipofuscin and other structures in the section (Fig. 4:12). Granules were stained in normal follicles in sections of the young horses between 3 to 7 years of age which lacked sufficient natural colour to be seen in H&E sections (Fig. 4:11 compared to Fig. 4:12). The granules were usually stained dark blue although in some

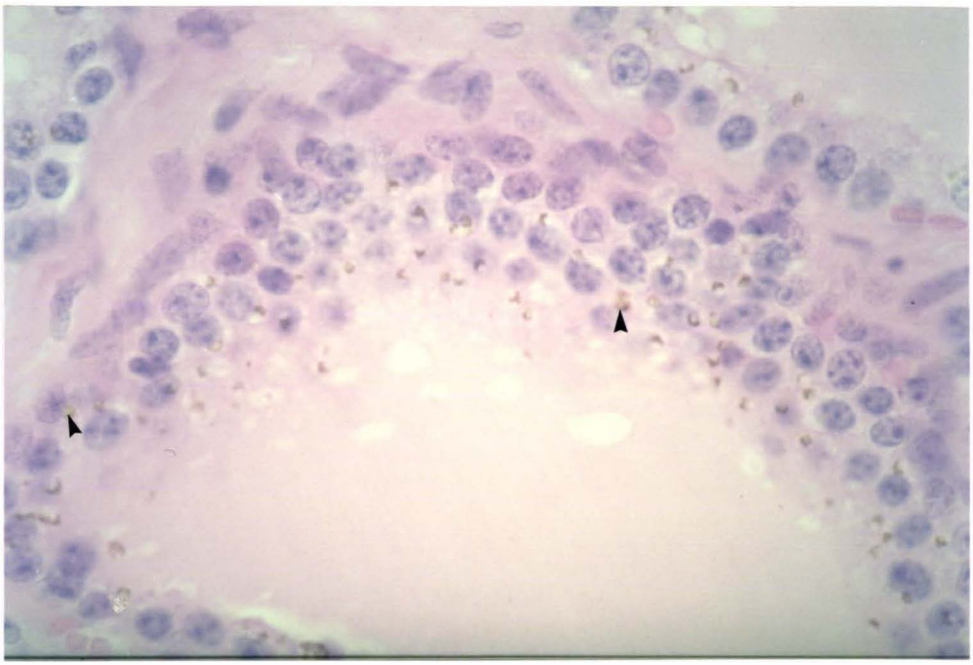
Figure 5:1

Lipofuscin granules by light microscopy. Although thyrocytes form a single layer of cells, in this section the follicle has been cut tangentially. Numerous lipofuscin granules with brown matrix and clear 'vacuoles' are present. Some vacuoles appear to have yellow contents (arrowheads). H&E x 400.

Figure 5:2

Thyroid tissue stained with PAS x 400.

The colloid and interstitial tissue stain intensely with PAS. Lipofuscin granules appear red or orange-red, with clear or yellow vacuoles. Some other cytoplasmic structures, the colloid, and the interstitial tissue are also stained.



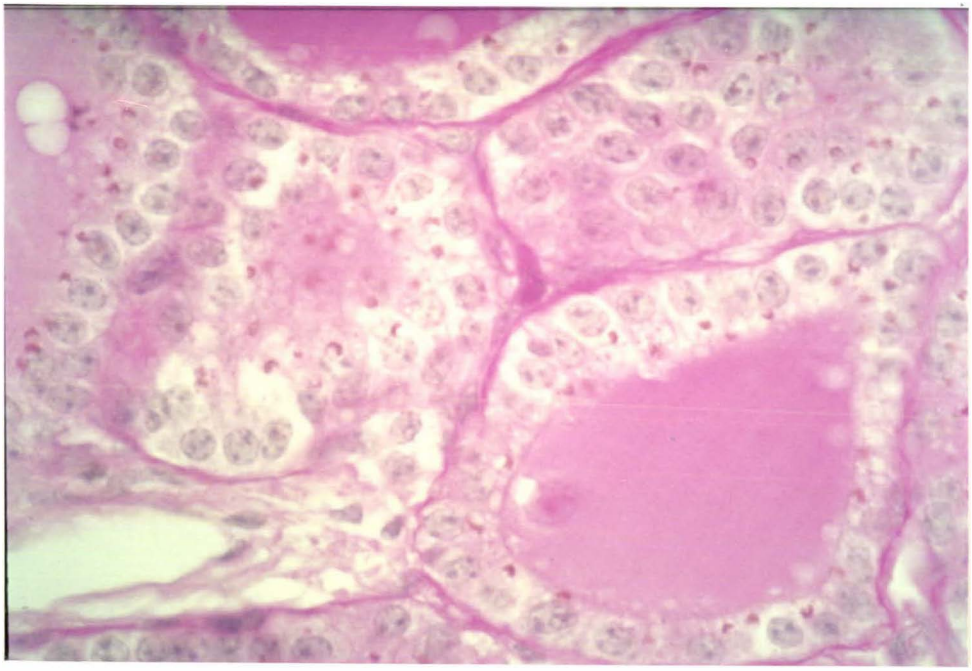


Table 5:1. Histochemistry of equine thyroid lipofuscin

Horse	PAS	Schmorl's	MF	AFIP	Perl's	Feulgen-NAH	MGP
PR	ngd	ngd	-	ngd	-	-	-
8M	+++++	++	+++	+++++	P.	-	-
1A	nil	+++++	-	+++++	-	-	-
3A	++	+++++	-	nil	-	-	-
3B	+++++	++	+++++	+++++	N.	-	-
5A*	++	++	+++	+++	P.	-	-
6B	+++	++	-	-	N.	-	-
6A	+++	++	-	-	N.	-	-
7A	+++	++++	-	-	N.	-	-
9A	+++	++++	-	-	N.	-	-
12A	nil	+++	-	-	N.	-	-
12B	+	+++	-	-	N.	-	-
12C	++++	+++	-	-	-	-	-
12D	++++	++++	++++	+	-	-	-
15A	nil	+	-	-	N.	-	-
15B	++++	+++++	-	-	N.	-	-
15C	+	++++	-	-	-	-	-
20A	+++	+	-	-	N.	-	-
20B	+	+	-	-	P.	-	-
20C	+	+++	-	-	P.	+	++++
20D	+++	+++++	+++		N.	-	-
20E	+	+++	-	-	-	-	-
20F	+++	+	++++	+++	-	-	-
25A	++	+++	-	-	-	+	+
25B	+++	+	-	-	-	-	-
25C	++++	++++	-	-	N.	-	-
25D	++++	+	-	-	N.	+	++
25E	+++	+++++	++++	+	-	-	-
25F	+++	+++++	++++	+++	-	-	-
25G	+	+	-	-	-	-	-
28A	+	+	-	-	-	-	-
28B	+++++	nil	++++	+++++	-	-	-
30A	+++	++++	++++	+++	-	-	-
30B	+++	++	++++	+	-	-	-
32A	++	++	+++	+	-	-	-
35A	+	+	-	-	-	-	-

Key and footnote:
ngd = no granules detected
- = stain was not used on a section from that horse.
nil = no reaction to stain
+ to +++++ = variation in stain intensity. At stain intensity +++++, all granules react strongly, with no native colour apparent.
P. = some granules in the section were Perl's-positive
N. = all granules in the section were Perl's-negative.

cases the natural colour showed through, resulting in some appearing dark grey or grey-brown. The stained lipofuscin clearly contrasted with the colloid and the cytoplasm, which stained pale blue, and cell nuclei, which stained pale pink with the counterstain. As with the PAS stain, the 'vacuoles' of granules did not stain with Schmorl's stain but were faintly stained by the counterstain. The intensity of staining varied between horses (Table 5:1). This variability was present between sections processed together, indicating that it was not related to the batch of stain used. In some individuals the affinity to Schmorl's stain was variable between granules of different follicles within the same section. There was no apparent relationship between stain affinity and age (Table 5:1).

Masson's Fontana stain: The lipofuscin matrix stained black with Masson's Fontana stain, in contrast to the 'vacuoles' which stained brown or did not stain at all (Fig. 5.3). The stained granules were in clear contrast to the pale pink counterstaining of the other structures in the section. However the detail of the other structures was poor using this stain relative to that in Schmorl's stained sections.

AFIP Stain for lipofuscin: Lipofuscin usually stained dark red with the AFIP stain for lipofuscin, which is a modified long Ziehl-Neelsen stain. The intensity of the reaction varied between horses (Table 5:1). The 'vacuoles' stained pale pink while intrusion of the natural colour of the lipofuscin frequently gave a red-brown or orange tinge to the matrix (Fig. 5:4).

Comparison of stain affinities: The staining characteristics of equine thyroid lipofuscin with a variety of stains are listed in Table 5:1. The affinities for different stains are not correlated. Lipofuscin in almost all individuals stained with PAS, Schmorl's and Masson's Fontana stains, as described above, and there was little or no variation in stain intensity within sections. This is in contrast to sections stained with Perl's stain for iron, Feulgen-NAH, Methyl Green-Pyronin and Oil Red O stains, as described below, in which variation in stain

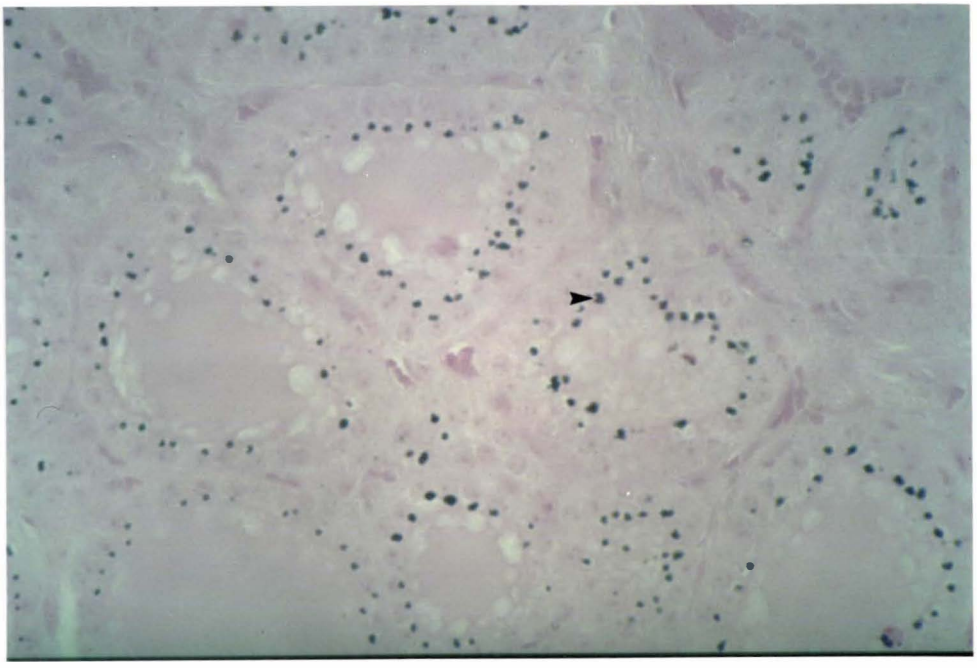


Figure 5:3

Thyroid tissue stained with Masson's Fontana x 100. Lipofuscin matrix stains intensely with this stain, but vacuoles vary from clear (arrowhead) to brown.

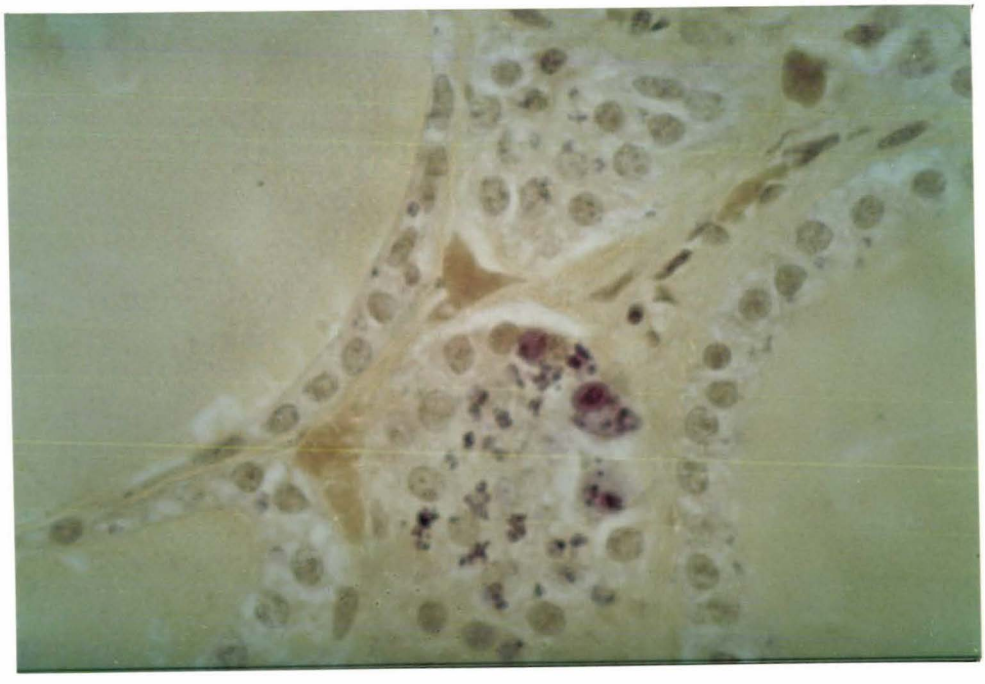


Figure 5:4

Thyroid tissue stained with AFIP stain for lipofuscin x 400. Acid-fast structures are normally stained red with this stain. Lipofuscin granules in this section are red-brown, due to intrusion of the natural colour.

intensity within sections indicated the presence of heterogeneous subpopulations of lipofuscin granules.

Perl's stain for iron: In sections from 4 of 17 horses, a few lipofuscin granules stained with Perl's stain (Table 5:1). These occurred in a minority of follicles which frequently exhibited abnormalities in the colloid. In most cases, Perl's-negative lipofuscin granules were also present in thyrocytes of the same follicle (Fig. 5:5).

Feulgen-NAH stain: The matrix of approximately 50% of lipofuscin granules in three sections stained yellow-green, suggesting the presence of RNA, which normally stains blue-green. However the colloid, which is primarily composed of the glycoprotein thyroglobulin, also stained blue-green. Some granules stained pink or red, consistent with presence of DNA (Fig. 5:6). This reaction was observed in the 'vacuole', rather than the granule matrix.

Methyl Green-Pyronin (MGP) Stain: Reaction of lipofuscin to this stain, which stains RNA red and DNA blue-green, varied between the three horses in which it was studied. In two horses a minority of granules stained a slight red while in a third an intense red reaction occurred in all granules (Fig. 5:7). All the sections were processed together. However the affinity for this stain was neither extinguished, nor clearly reduced, by incubation in RNAse, 1 ug/ml, prior to staining. Colloid was also slightly to moderately stained.

Lipid stains: Lipofuscin reacted moderately to Sudan Black B in both frozen sections and paraffin sections, but staining did not extend to the 'vacuoles' (Fig. 5:8). The great majority of granules did not stain with Oil Red O in frozen sections (Fig. 5:9) although occasionally a faint reaction could be observed in a 'vacuole'.

Concanavalin A-peroxidase labelling: The matrix of the majority of lipofuscin granules, in sections from the three horses examined, reacted with the Concanavalin A-peroxidase label, indicating the

Figure 5:5

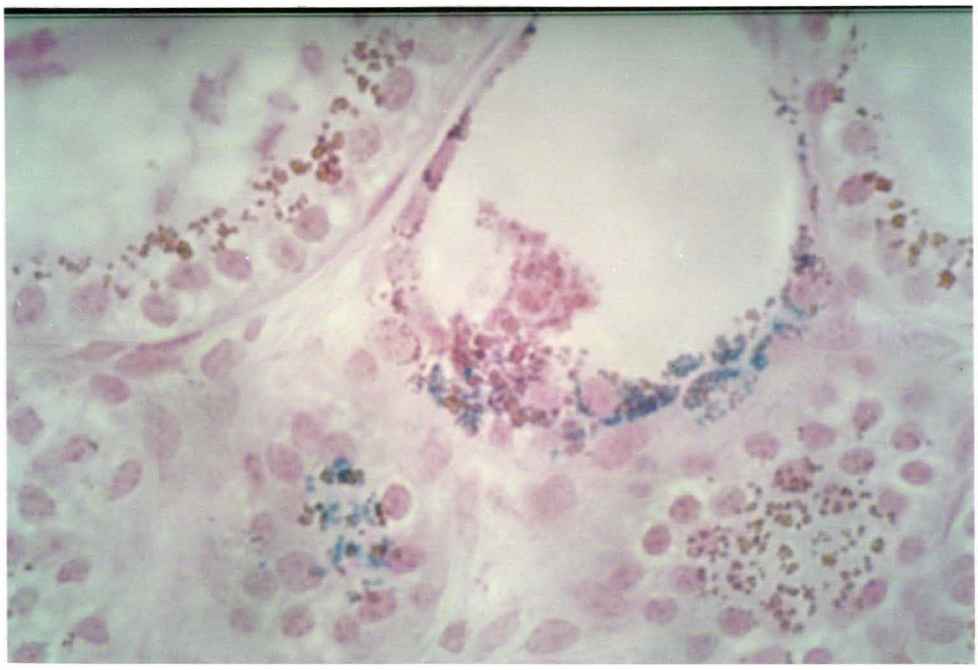
Thyroid tissue stained with Perl's stain for iron x 400

The lipofuscin of most follicles remains a brown colour. However in occasional follicles some granules show a positive blue reaction for iron.

Figure 5:6

Thyroid tissue stained with Feulgen-NAH stain x 400.

Some lipofuscin vacuoles show a red positive reaction for DNA, particularly in the granule matrix. The matrix of some granules appears greenish, suggesting the presence of RNA. However the colloid also stains blue-green, a false positive reaction for RNA.



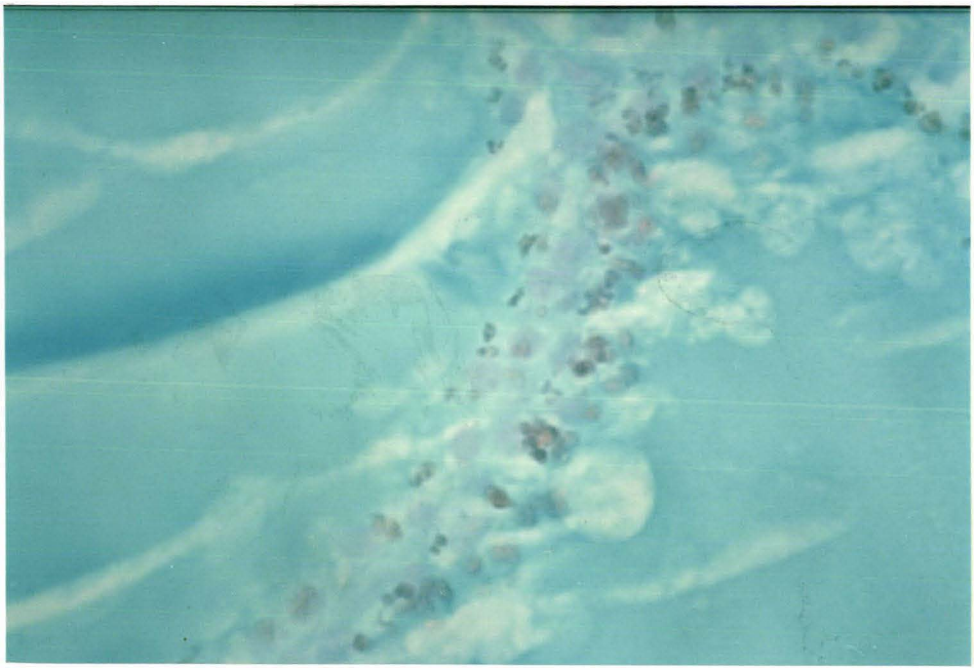
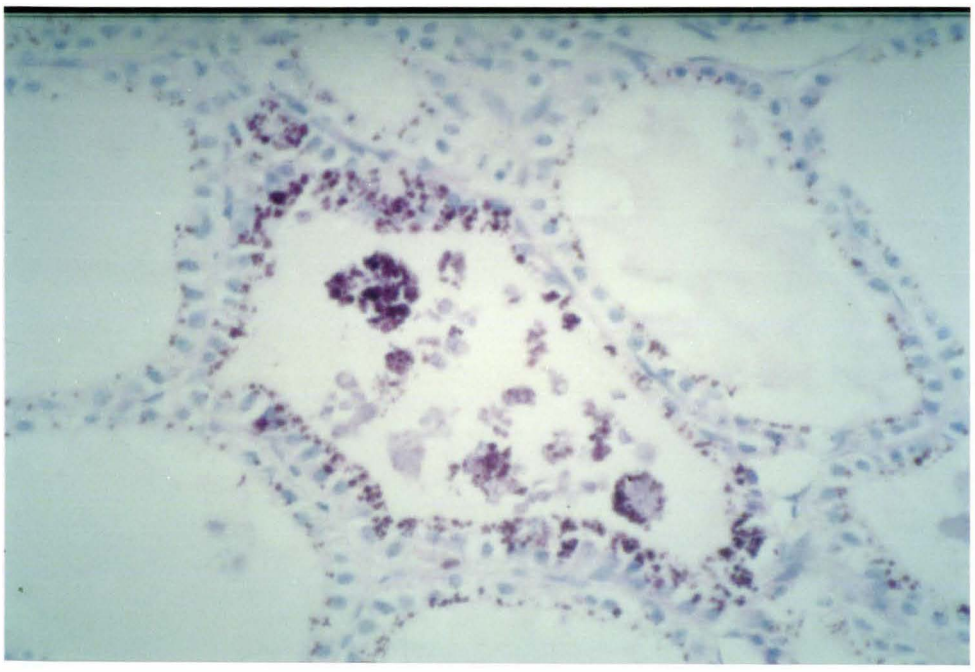


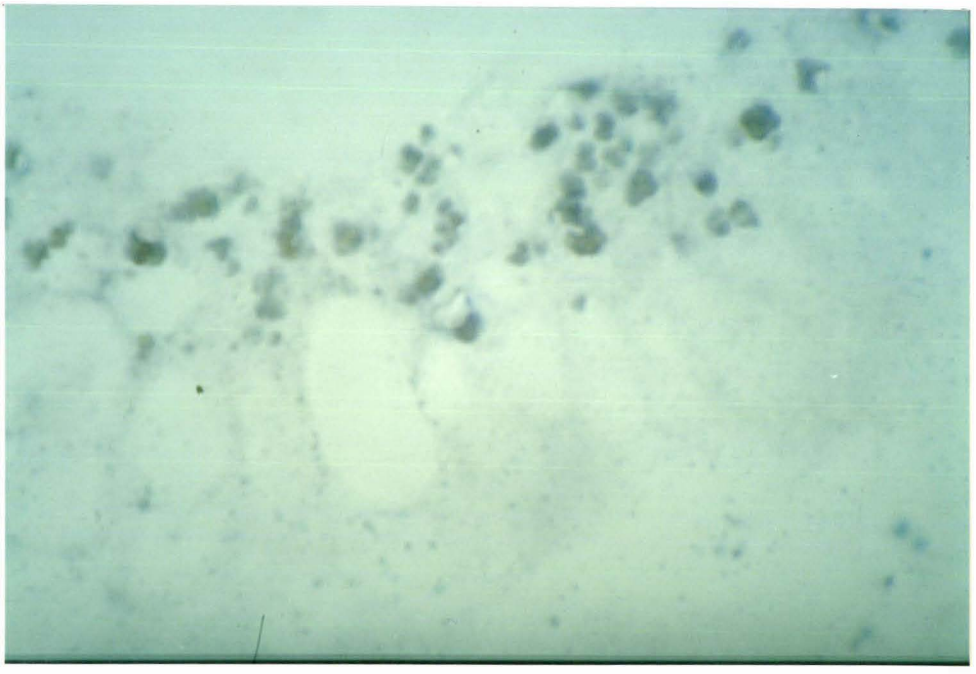
Figure 5:7

Thyroid tissue stained with Methyl Green-Pyronin (MGP) x 100. Lipofuscin granules in thyrocytes and spherites show a strong positive reaction to this stain. However this reaction was not extinguished by prior incubation with RNAse, and the colloid and cytoplasm show a false positive reaction.

Figure 5:8

Frozen section of thyroid stained with Sudan Black B. Lipofuscin granules show partial staining of the matrix only. Lipofuscin 'vacuoles' are not stained, and cell nuclei are not stained. x 1000.





presence of mannose and/or glucose (Fig. 5:10). The natural yellow colour of the granules was not always masked by the label. Granule 'vacuoles' were not labelled. Some cells showed heavy cytoplasmic labelling which did not appear to be associated with lipofuscin. Follicular colloid was moderately labelled.

Histochemistry of lipofuscin in the 5 year old horse

The exceptionally large granules in the thyrocytes of the abnormal follicles in the 5 year old horse (Fig. 4:13) exhibited differences in histochemistry compared to the granules in the thyrocytes of normal follicles in the same section. They showed a greater PAS affinity, but were less Schmorl's-positive and did not stain with Masson's Fontana stain.

Ultrastructure of equine thyroid lipofuscin

The ultrastructure of thyroid tissue of horses from 9 to 35 years of age was examined. There was no age-related changes in the ultrastructural appearance of the thyroid tissue, the morphology or size of the granules, or their apparent interactions with colloid droplets.

Lipofuscin granules were typically located in the apical cytoplasm (Fig. 5:11), and were irregular in shape. They were observed in similar numbers to those observed by light microscopy in the same horses. The matrix was electron-dense and the 'vacuoles' relatively more electron-lucent. The 'vacuole' contents were homogeneous and of variable electron-density (Fig. 5:12). In some cases they were nearly as electron-dense as the matrix material (Fig. 5:13), but more commonly they were slightly less electron-dense than the extracellular colloid. Vacuoles were found both deeply embedded in matrix and in peripheral locations, bulging out from the granules. The periphery of the vacuole was outlined by an irregular layer of matrix or a layer of tiny vacuoles (Fig. 5:14). Vacuoles were usually round or oval, but large vacuoles were sometimes bean-shaped rather than circular in section, suggesting that the matrix had broken down between two vacuoles of similar size and they had coalesced into one (Fig. 5:15). Small vacuoles were in some cases

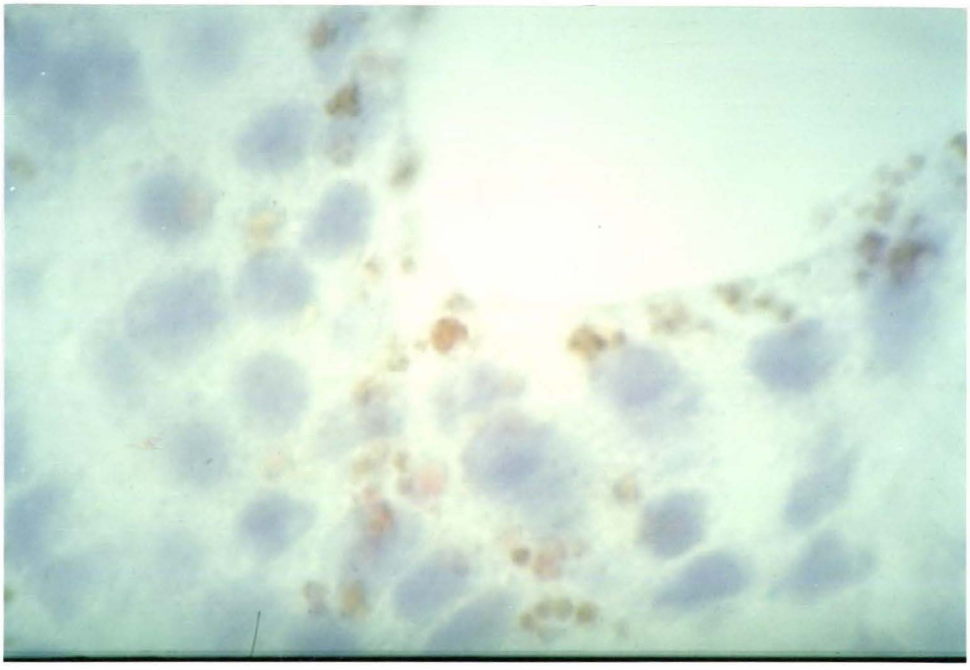
Figure 5:9

Frozen section of thyroid tissue stained with Oil Red O. Only rarely do lipofuscin granules show a faint red colour (bottom centre). x1000

Figure 5:10

Thyroid tissue labelled with Concanavalin A/Horseradish peroxidase x 1000.

The matrix of lipofuscin granules is labelled, although the yellow colour frequently intrudes. The thyroglobulin in the colloid pool, which is a glycoprotein containing mannose, shows a moderate positive reaction.



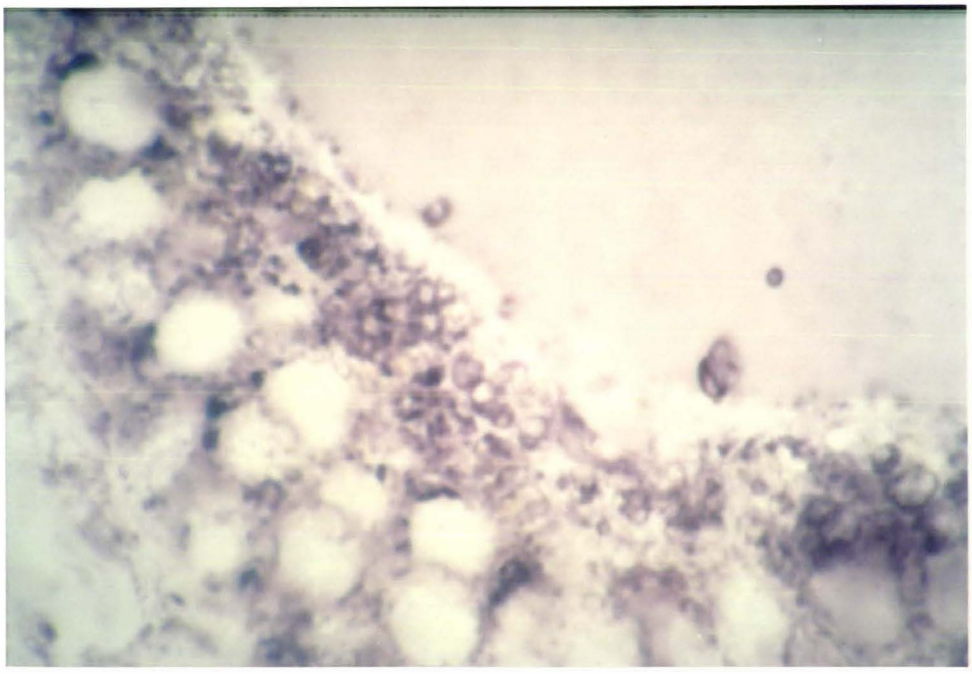


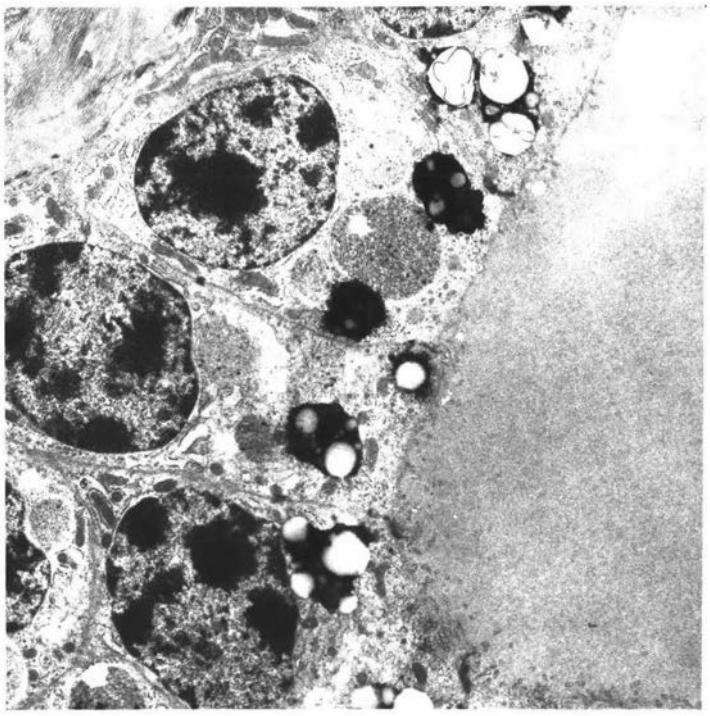
Figure 5:11 x 5000

Ultrastructure of equine thyroid tissue. The colloid is at right. Some interstitial connective tissue is at upper left. The apical cytoplasm of the thyrocytes contains lipofuscin granules of various sizes, with electron dense matrix and vacuoles of variable size and electron density. Tears in the vacuole contents are present in the lipofuscin at the top of the picture. Colloid droplets of variable electron density are also present in the apical cytoplasm.

Figure 5:12 x 11,000

Variations in electron density of lipofuscin and colloid droplets.

The thyrocyte at centre has been sectioned tangentially, excluding the cell nucleus. The cytoplasm contains two lipofuscin granules, with vacuole contents similar to, or denser than, the colloid at lower right. A number of colloid droplets are present in the same part of the cell. The contents of these vary in density and include clear areas and electron-dense particles.



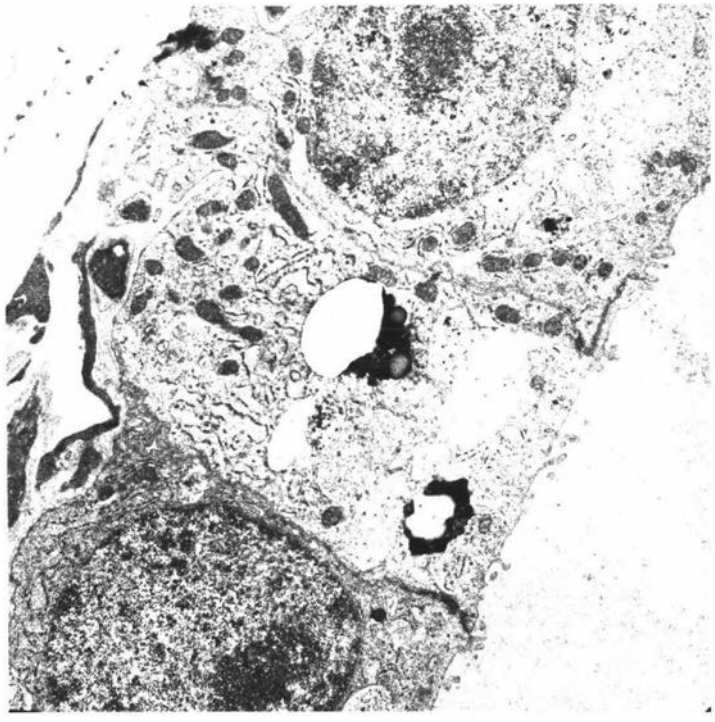


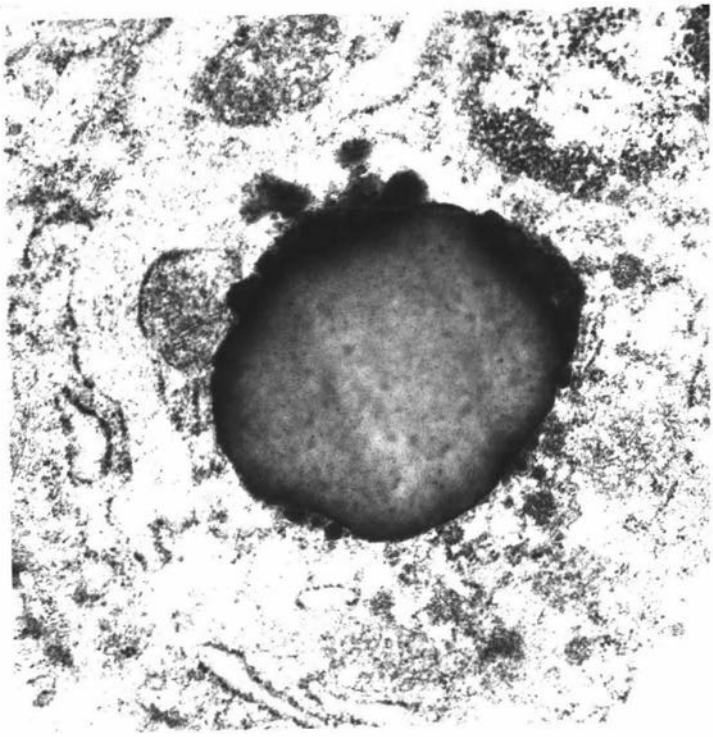
Figure 5:13 x 32,000

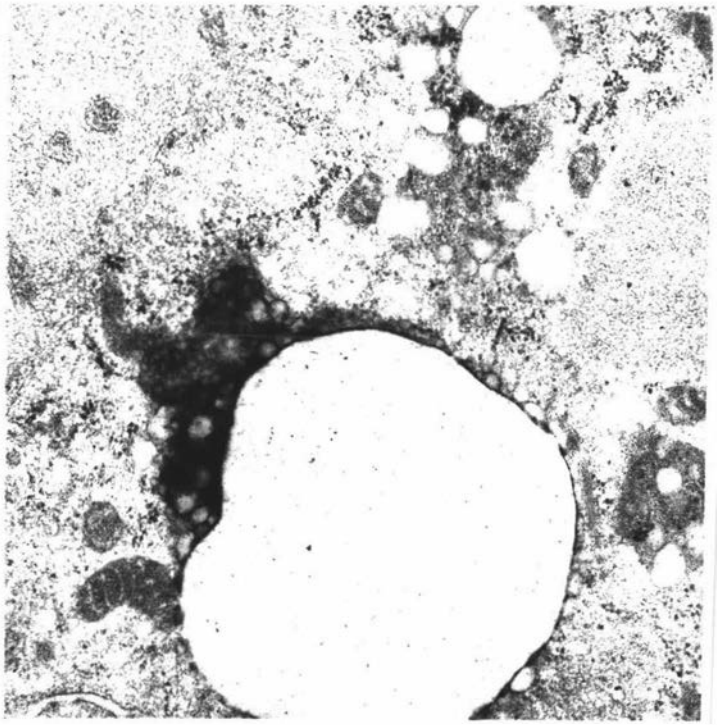
Lipofuscin granule with vacuole of high electron density.

Figure 5:14 x 32,000

Fine detail of lipofuscin granules.

The lipofuscin granules contain numerous vacuoles of different sizes. The largest vacuole of the granule at bottom centre is surrounded by an electron-dense layer and a layer of very small vacuoles. The matrix of the granules at upper right and lower right shows unusually low electron density.





apparently continuous with larger vacuoles, suggesting that they were coalescing (Fig. 5:16).

Vacuoles were observed in most sections in which the contents contained holes or tears, with vacuole contents shrinking back from the edges of the hole (Fig. 5:11, Fig. 5:16). The resin of the section was intact.

The fine detail of the matrix was granular. Lamellar patterns were not observed.

Lipofuscin granules were usually found close to colloid droplets (Fig. 5:11, Fig. 5:12). The electron-density of colloid droplets was variable but was usually less than that of the colloid in the follicular lumen. In several cases the vacuolar part of the lipofuscin appeared to be fused with the lumen of an adjacent colloid droplet (Fig. 5:15).

Some colloid droplets contained electron-dense structures strongly resembling small lipofuscin granules with glassy 'vacuoles' embedded in a matrix (Fig. 5:17).

In order to determine whether the electron-density of the matrix component of the lipofuscin was intrinsic or due to osmiophilia and/or staining, the granules were examined in an unstained section from a tissue block processed without postfixing in osmium tetroxide. The granule matrix was still the darkest structure in the cell (Fig. 5:15). Resolution of cellular detail in such unstained sections was predictably poor. Examination of tissue from the same horse processed with osmium tetroxide fixation showed that electron-density of both parts of the granules was enhanced by osmium. The matrix was also further stained by uranyl acetate and lead citrate in addition to its intrinsic electron-density.

Immunogold electron microscopy

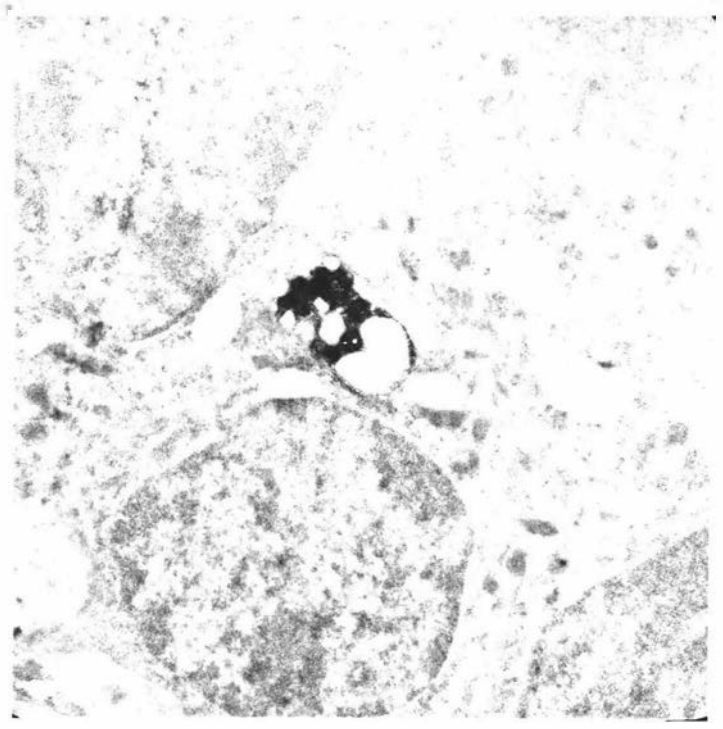
The immunogold probe using antibody raised in rabbits against equine thyroglobulin reacted strongly with the follicular colloid, which

Figure 5:15 x 11,000

Thyroid tissue processed without osmium and not stained. The colloid is at upper right. Three cell nuclei are partially included in the figure. The matrix of the lipofuscin granule (centre) has the highest natural electron-density of the structures present. Some of the vacuoles in the granule exhibit some electron-density. The clear areas in the cytoplasm of the thyrocytes are most likely distended cisternae of rough endoplasmic reticulum, although some may be colloid droplets.

Figure 5:16 x 15,000

Apparent fusion between a lipofuscin granule and a colloid droplet, centre. Small vacuoles are apparently coalescing with larger ones in the lipofuscin granule at top. The lower lipofuscin granule contains a vacuole (far left) with a tear in the contents.



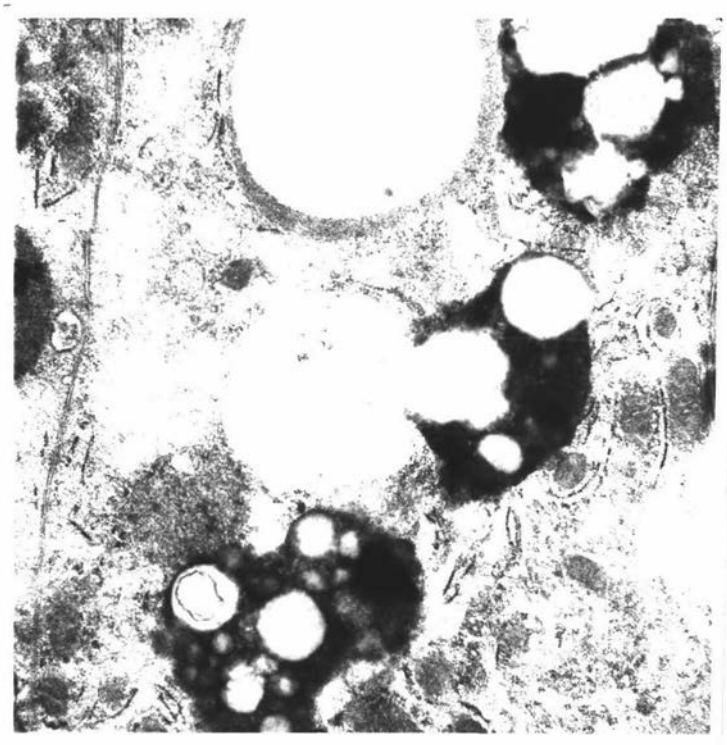


Figure 5:17 x 11,000

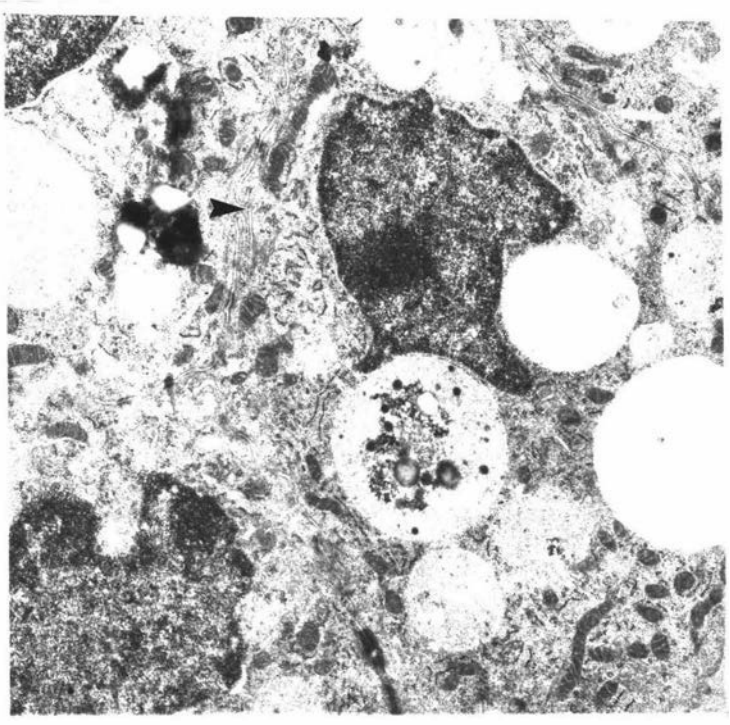
Variations in contents of colloid droplets.

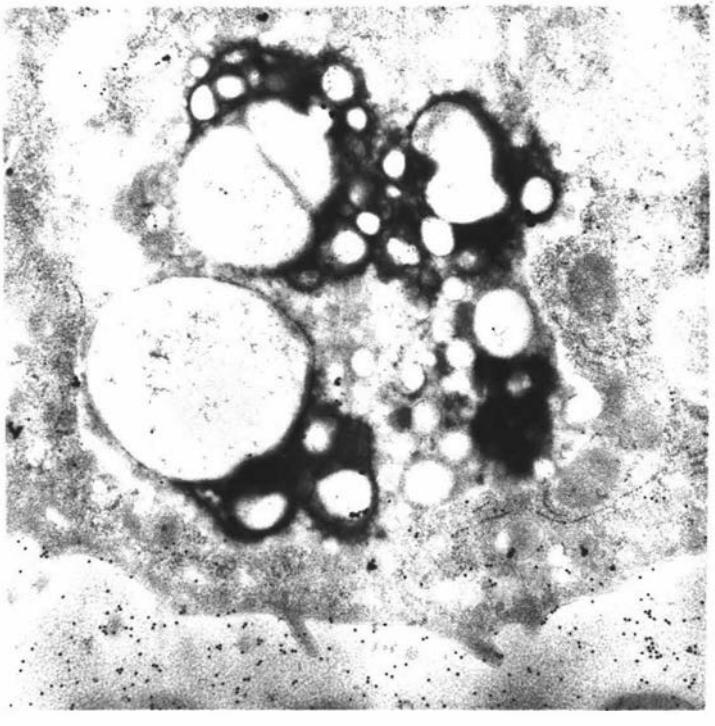
Numerous colloid droplets with contents of variable density are present in these cells. Colloid droplet contents include clear areas, strands of material, electron-dense particles and, in the droplet at centre, structures strongly resembling the vacuoles of lipofuscin granules.

Extensive interdigitations are present in the cell membranes between thyrocytes (arrowhead).

Figure 5:18 x 21,000

Anti-thyroglobulin immunogold labelled thyroid tissue. The extracellular thyroglobulin (the colloid) provides a positive control. The cytoplasm shows light labelling. The lipofuscin granules show negligible labelling. Colloid droplets (upper right, centre right) are also unlabelled. The large vacuole at upper right is bean-shaped, suggesting that two vacuoles may have coalesced into one. A small vacuole appears to be continuous with the large vacuole at upper left.





confirmed that the antibody raised was against thyroglobulin. The probe also reacted lightly with the cell cytoplasm. Lipofuscin granules and cell nuclei did not react with the antibody (Fig. 5:18). Only a minority of colloid droplets were labelled.

DISCUSSION

Histochemistry of lipofuscin

The lipofuscin granules were recognized by their natural colour, fluorescence and histochemical reactions (see Introduction), but considerable heterogeneity was observed in their histochemistry. Granules were most easily seen by fluorescence microscopy or when stained with Schmorl's or Masson's Fontana stains.

The lipofuscin granules in H&E-stained sections varied in colour. The granules appeared more brown in H&E sections than in unstained serial sections from the same individuals, indicating that some staining occurred. However some granules detected by their natural colour in unstained sections could not be seen in H&E stained serial sections. This was most noticeable in horses 3A, 3B and 5A, but was also observed in some individuals up to the age of 20. It has been reported that lipofuscin has little or no colour when newly formed, and develops colour with time (Few and Getty 1967, Dolman and Macleod 1981, Pearse 1985). The granules which were lacking in sufficient natural colour to stand out in H&E stained sections may be newly-formed, 'immature' granules, but they were found in horses of all ages.

The intensity of the reactions with most of the histochemical stains was variable between horses, indicating variation in the reacting groups in the lipofuscin. Variation in staining affinity within a section was observed with some stains, indicating the existence of heterogeneous subpopulations of granules. There was no apparent relationship between affinity for any histochemical stain and age. According to Pearse (1985), lipofuscin gains reducing capacity with 'maturation'. If this were so, and assuming that lipofuscin granules are long-lived structures, the reaction with Schmorl's and Masson's

Fontana should be greater in the older horses. If a trend towards increasing reducing capacity existed, it should be apparent from this study because the ages of the horses spanned 35 years, which is close to the maximum usual lifespan in this species. Either the granules are not long-lived, or the reducing capacity of lipofuscin granules does not increase with 'maturation' in this tissue.

PAS is a stain for carbohydrates and hence stains glycoproteins such as the thyroglobulin in the follicular colloid. The positive reaction with Concanavalin A demonstrates the presence of mannose and/or glucose in equine thyroid lipofuscin, and the positive PAS reaction is therefore most likely to be due to sugars. The reaction of periodic acid with sugars produces aldehydes which then react with Schiff's reagent to produce Schiff bases. The positive reaction of lipofuscin with PAS has been attributed to the presence of aldehydes which are not derived from carbohydrates (Pearse 1985), on the apparent assumption that lipofuscins do not contain carbohydrates. Malondialdehyde and other aldehydes alleged to be produced by lipid peroxidation reactions could theoretically react with Schiff's reagent in this stain, but there is no reason to attribute the positive reaction in this lipofuscin to pre-existing aldehydes rather than carbohydrates.

Equine thyrocyte lipofuscin was most effectively stained with Schmorl's and Masson's Fontana stains. The intensity of the reaction with Schmorl's stain varied between individual horses. A positive reaction to this stain indicates that ferric ferricyanide has been reduced. Therefore heterogeneity existed between horses in the reducing capacity of the lipofuscin, but the reducing groups responsible for the reaction are unknown (Pearse 1985). The extracellular colloid also reacted slightly to this stain, staining a much paler blue. There was evidence from the ultrastructural observations that the contents of colloid droplets contribute to lipofuscin granules (Fig. 5:15). The same reducing groups, such as those of reducing sugars from thyroglobulin, might be responsible for the positive reaction in colloid and lipofuscin. The much darker colour in the lipofuscin, reflecting a greater reductive capacity,

could be indicative of a higher concentration of reducing groups. The lack of correlation between reaction to PAS stain and reaction to Schmorl's stain (Table 5:1) suggests that the stains are reacting with different chemical groups, so it is unlikely that both reactions are wholly attributable to the presence of reducing sugars.

The Masson's Fontana stain, like Schmorl's stain, indicates the presence of reducing capacity. It utilises the 'argentaffin reaction' in which ionic silver in basic solution is reduced to metallic silver (Pearse 1985). Landas et al (1986) used affinity for Masson's Fontana as a criterion for classifying pigmented granules in thyrocytes as 'neuromelanin' or as 'lipofuscin', but the stain is not specific for melanin, and is known to stain lipofuscin in other tissues (Stevens 1968, Lillie 1969, Pearse 1985). However it is not clear from the literature how positive or consistent the reaction is. The reaction of equine thyroid lipofuscin may be unusually strong compared to that of other lipofuscins. If not, it is surprising that Masson's Fontana is not frequently recommended as a stain for lipofuscin. The argentaffin reaction is of low chemical specificity, and for practical purposes a positive reaction suggests the presence of phenols (Pearse 1985). The reducing capacity of melanin has been attributed to the presence of substituted phenols (Barden and Brizzee 1987) derived from tyrosine. The iodination of tyrosine to form the iodothyronines results in phenols with iodine atoms on the ring, which would stabilise the oxidised form. Such molecules might react in the same way as the substituted phenols in melanin. Colloid, which contains iodothyronines, does not stain with Masson's Fontana, but lipofuscin could contain greater concentrations of iodothyronines or iodothyronine-derived molecules with greater reactivity. Pigmented, oxidised derivatives can be made in vitro from dopamine, epinephrine and norepinephrine, which like melanin are formed from tyrosine via dihydroxyphenylalanine (DOPA) (Hack and Helmy 1983). It is possible that iodothyronines could undergo similar changes, and that such derivatives, if they exist, could be responsible for the reducing capacity and the natural colour of equine thyroid lipofuscin. However this is speculation, and no conclusions can be drawn.

The 'vacuoles' in lipofuscin granules have been assumed to contain lipid (Borel and Reddy 1973, Matsubara et al 1982, Alexander et al 1985). Lipid would be removed during processing of paraffin-block sections, but the contents of the vacuoles were preserved as pale yellow areas in H&E and PAS stained sections. As further evidence that the 'vacuoles' were not lipid, they did not stain with Sudan Black B, and only rarely gave a weak partial reaction with Oil Red O, in frozen sections. Furthermore the 'vacuoles' occasionally showed a positive Feulgen-NAH reaction for DNA.

A positive reaction of thyroid lipofuscin to Perl's stain for iron was observed in a subpopulation of granules in only four individuals, usually in thyrocytes of follicles containing abnormal colloid. Thyrocytes will endocytose erythrocytes in colloid (Wollman 1980), and Perl's-positive granules could be a product of lysosomal catabolism of such erythrocytes followed by sequestration of the iron from them. Analysis of the metal content of isolated lipofuscin showed wide variation in the iron content between individuals (see Chapter 6).

Two other stains which stained only some lipofuscin granules were Feulgen-NAH stain and Methyl Green-Pyronin stain, both stains for nucleic acids. The demonstration of DNA by the use of Feulgen-NAH stain was a surprising finding in a cytoplasmic structure. Theoretically mitochondrial DNA could enter the lysosomal system by autophagy, but the DNA in isolated lipofuscin was shown to be nuclear DNA (see Chapter 6). There is very little information in the literature on the dynamics of nucleic acid catabolism in vivo. Although nucleases are abundant in many tissues, it is believed that DNA is broken down very slowly, if at all, in cells during life (Davidson 1972). If this is so, then the nuclear DNA in lipofuscin was most likely to be derived from the endocytosis of the remains of thyrocytes which had exfoliated into the colloid. The positive reaction for DNA observed in the vacuoles, which apparently fuse with colloid droplets, is consistent with this.

RNA stains blue-green with Feulgen-NAH stain, but the lipofuscin stained yellow-green. This colour difference could be due to intrusion of the natural colour of the granules. Although lipofuscin stained with Methyl Green-Pyronin, the reaction was not substantially reduced in the RNAse-treated control, so the presence of RNA cannot be confirmed by this result. Methyl Green-Pyronin stain is not specific for RNA (Pearse 1985). If RNA is present, it could originate from ribosomes which enter the lysosomal system by autophagy. Alternatively ribosomes or their remains could be endocytosed with other debris of cells exfoliated into the colloid. Analysis of the phosphorus content of isolated lipofuscin, and comparison with the DNA content, showed that only traces of RNA can be present in lipofuscin (see Chapter 6).

The histochemical differences observed between lipofuscin granules in thyrocytes of normal follicles and those in thyrocytes of abnormal follicles of the 5 year old horse indicate that the latter are a distinct subpopulation. They may have a different chemical composition, possibly as a result of changes in the contributing substrate. Alternatively the granules of the abnormal follicles may have differed in chemical composition from the granules of the normal follicles because they were at a different stage of 'maturation'. Such histochemical diversities were not observed in the 6 year old horses, which also had particularly large lipofuscin granules in abnormal follicles, but if gradual catabolism of lipofuscin was occurring, the diversity might slowly disappear.

Ultrastructure

The ultrastructure of equine thyroid tissue was in accord with published studies of other species (Heimann 1966, Lupulescu and Petrovici 1968, Klinck et al 1970, Ives et al 1975).

Equine thyroid lipofuscin showed the same ultrastructural features as those described in other species, including irregular shape (Heimann 1966, Lupulescu and Petrovici 1968, Alexander et al 1985), variable size (Heimann 1966, Ives et al 1975, Matsubara et al 1982), an electron-dense matrix (Lupulescu and Petrovici 1968, Alexander et al

1985) and electron-lucent vacuoles (Heimann 1966, Lupulescu and Petrovici 1968, Klinck *et al* 1970). The apparent fusion of colloid droplets to lipofuscin (Fig. 5:15), suggests that lipofuscin retains some lysosomal function, and supports the conclusion that the contents of lipofuscin are products of endocytosis. There is no ultrastructural evidence that autophagy or crinophagy contribute to lipofuscin granules.

Colloid droplets containing particulate inclusions resembling lipofuscin granules, as reported in the cat (Ives *et al* 1975), were also found in the thyroid of the horse (Fig. 5:16). It is likely that these particulate inclusions represent an early stage in the biogenesis of lipofuscin granules (Ives *et al* 1975). Klinck *et al* (1970) reported 'structures intermediate between typical cytosomes and typical lipofuscin granules' in human thyroid, which he considered to be transitional stages between the two. Heimann (1966) and Lupulescu and Petrovici (1968) both identified lipofuscin granules in electron micrographs as 'phagolysosomes'. Colloid droplets are also a type of phagolysosome.

The high natural electron-density of granules processed for electron microscopy without osmium and examined without staining indicates the presence of elements of high atomic weight, probably metals and/or iodine.

Given the evidence that colloid droplets probably contribute to lipofuscin granules, the granules may be composed, at least in part, of products of thyroglobulin catabolism. The molar ratios of amino acids in thyrocyte lipofuscin are similar to those in thyroglobulin (see Chapter 6). *In vitro* digestion with enzymes extracted from thyroid lysosomes has been shown to destroy the antigenic determinants of thyroglobulin (Tokuyama *et al* 1987). Therefore the lack of labelling by the anti-thyroglobulin immunogold probe may reflect lysosomal catabolism of the antigenic determinants. Most of the intracellular colloid droplets were also not labelled by the immunogold probe, in contrast to the strongly labelled extracellular colloid. Fusion with lysosomes occurs within 5 to 10 minutes of

droplet formation (Wollman 1980, van den Hove-Vandenbroucke 1980), and most colloid droplets observed in this study were less electron-dense than the extracellular colloid. This is a characteristic of colloid droplets that have fused with lysosomes and are acid phosphatase-positive, in contrast to newly-formed, acid phosphatase-negative colloid droplets which are more electron-dense than colloid (Wollman 1980). Therefore it is likely that most colloid droplets observed already contained lysosomal enzymes, which had catabolised the antigenic determinants of the thyroglobulin. Alternatively thyroglobulin fragments may adopt a tertiary structure which makes the antigenic determinants inaccessible to antibody.

A similar finding has been reported in another lipofuscin. Despite strong evidence that shed photoreceptor outer segment discs contribute to lipofuscin in human retinal pigment epithelial cells, rhodopsin cannot be demonstrated in this lipofuscin by immunocytochemistry (Feeney-Burns et al 1988), although it can be demonstrated in secondary lysosomes from the same cells. It is probable that the rhodopsin is not recognized because it has undergone partial lysosomal catabolism.

SUMMARY

There was considerable heterogeneity in the histochemistry of equine thyroid lipofuscin, but this was not related to age.

The lectin histochemistry demonstrated the presence of mannose and/or glucose in most lipofuscin granules. The positive PAS reaction of this lipofuscin was attributed to sugars rather than to peroxidation-derived aldehydes.

Lipofuscin granules in equine thyroid characteristically stained well with stains which demonstrate reducing groups.

There was histochemical evidence for the presence of iron in some lipofuscin granules and DNA in others.

Histochemical heterogeneity between lipofuscin granules within the same follicle (Fig. 5:5, Fig. 5:6, Fig. 5:8, Fig. 5:9), and between granules in different follicles, indicated that distinct subpopulations, possibly arising from diverse substrates entering the lysosomal system, exist within thyrocyte lipofuscin.

Equine thyroid lipofuscin stained poorly for lipids even in frozen sections. There was no histochemical evidence to support the alleged lipid nature of the 'vacuoles'.

The ultrastructural study supports the hypothesis that lipofuscin granules are active organelles derived from lysosomes that fuse with colloid droplets. As such, the traditional term 'granule' has some misleading connotations. However, for simplicity, the use of the term will continue in this thesis.

The negative reaction of the granules with an anti-thyroglobulin immunogold probe does not negate the hypothesis that endocytosed colloid contributes to lipofuscin.

CHAPTER 6
LIPOFUSCIN IN THE EQUINE THYROID GLAND: ISOLATION AND CHEMICAL ANALYSES

INTRODUCTION

Quantitative analytical studies of lipofuscin have rarely been attempted. Notable exceptions are the analyses of human cardiac lipofuscin by Hendley et al (1963a,b), and Bjorkerud (1964). The former reported a lipid content of up to 40% w/w, while the latter identified a wide variety of lipid species which made up 51% of the lipofuscin. Proteins were found in both studies, and Bjorkerud (1964) found that these represented 30% of the lipofuscin. In addition, Hendley et al (1963a,b) found an iron content up to 0.2%. The widely held belief that lipofuscin is principally derived from peroxidation of lipid, with oxidative damage to proteins possibly involved as a secondary phenomenon (see Introduction), has been deduced from observation of its fluorescent and histochemical properties and from theoretical arguments.

The histochemistry of equine thyroid lipofuscin suggested the presence of several different chemical species. The affinity for lipid stains was weak. The positive reaction to Concanavalin A indicated the presence of mannose and/or glucose residues, and the positive PAS reaction also supported the presence of carbohydrate. There was histochemical evidence for the presence of iron and nucleic acids in some granules.

Based on the histological and ultrastructural evidence, the thesis has been developed in the two preceding chapters that lipofuscin granules in equine thyrocytes are an intermediate stage of functional lysosomes, and that at least some of the chemical species contained in them are derived from endocytosed colloid.

This chapter reports the isolation, characterisation, and chemical analyses of isolated lipofuscin.

SPECIAL METHODS

Solubility of lipofuscin protein

A variety of solvent systems were explored, as set out below, to see which best dissolved lipofuscin protein.

1) Pellets of freshly isolated lipofuscin were mixed with aqueous solutions of 1-3% lithium dodecyl sulphate (LDS) with 0-15% 2-mercaptoethanol, with and without incubation at 90^{o}C for 5 minutes. The effect of pH on the solubility of lipofuscin in these solutions was also tested at pH 7 and at pH 10, using lithium hydroxide to raise the pH. The extent to which the protein was soluble was tested by quantitative amino acid analyses of the soluble and insoluble fractions of lipofuscin, and an aliquot of the lipofuscin that had not been exposed to detergent.

2) The solubility of fresh lipofuscin in formic acid was tested under a range of conditions as follows.

a) 90% formic acid and sonication at full power (see Chapter 3) for 60 seconds.

b) 90% formic acid + 1% 2-mercaptoethanol and sonication at full power for 60 seconds.

c) 98-100% formic acid and sonication at full power for 60 seconds.

d) 98-100% formic acid + 1% 2-mercaptoethanol and incubation for 18 hours at 37^{o}C with continuous mixing.

The insoluble fraction of the lipofuscin was pelleted by centrifugation.

3) The following experiments were carried out to determine whether lipofuscin could be made soluble in a solution of acid and detergent, and then remain soluble as the solution was adjusted to neutral pH.

a)A lipofuscin pellet was resuspended in 10x (v/v) 90% formic acid, and 1% LDS added. The mixture was titrated to pH 7 with ammonia and the insoluble fraction was pelleted by centrifugation.

b)A lipofuscin pellet was resuspended in 10x (v/v) 90% formic acid, and 3% Triton X-100 added. The mixture was dried under vacuum over potassium hydroxide (KOH).

c)A lipofuscin pellet was resuspended in 10x (v/v) 90% formic acid, and 2.5% (w/v) N-Tetradecyl-N,N-dimethyl-ammonio-3-propane sulphonate added. The mixture was dried under vacuum over KOH.

4) A further attempt at partial separation was carried out by the addition of 1 ml 0.1 M Tris HCl, pH 8.5 in 7.5 M guanidine HCl, to 1 mg lipofuscin. 2-mercaptoethanol, 15 ul, was added, the sample incubated for 5 minutes at 100^{o}C and incubated at 37^{o}C for 2 hours. The insoluble fraction was pelleted by centrifugation.

Control thyroid extracts for lithium dodecyl sulphate polyacrylamide gel electrophoresis (LDS-PAGE)

Control thyroid extracts were obtained from horses up to 5 years of age, as these thyroids were unlikely to contain large amounts of lipofuscin. A sample of total thyroid homogenate from an 18 month old filly was collected following homogenisation in a Sorvall Omnimixer (Ivan Sorvall Inc.) for 45 seconds at full speed. Total thyroid homogenates from two young horses, a yearling colt and a 5 year old gelding, were separated into water-soluble supernatants and water-insoluble pellets by centrifugation for 30 minutes 1500 g max at 4^{o}C. Samples of supernatants and pellets were collected. The pellets were resuspended in distilled water, and all samples were mixed with an equal volume of 6% LDS/20% 2-mercaptoethanol and incubated at 90^{o}C for 5 minutes.

Dry matter content of equine thyroid

Fresh thyroid tissue was weighed, lyophilised, weighed again, and the dry matter content calculated.

Other methods are given in Chapter 3.

RESULTS

Dry matter content of equine thyroid

The mean dry matter content of equine thyroid tissue was 25.6% (Table 6:1). This result was used for subsequent calculations of the yield of lipofuscin from thyroid tissue.

Table 6:1. Dry matter content of equine thyroid tissue

Horse age/sex	Wet weight (g)	Dry weight (g)	Dry matter content
2/g	1.04	0.30	28.8%
4/g	1.00	0.24	24.0%
4/g	1.05	0.29	27.6%
11/f	1.75	0.48	27.4%
20/s	1.50	0.35	23.3%
20/g	1.03	0.26	25.2%
20/f	1.03	0.24	23.3%
25/g	1.41	0.34	24.1%
32/f	1.12	0.30	26.8%
30/g	2.08	0.46	22.1%
30/f	1.17	0.34	29.1%
		Mean =	25.6% s.d. = 2.4

Key:
s = stallion
g = gelding
f = female

Isolation of lipofuscin

Isolation of lipofuscin granules was achieved without recourse to the use of proteolytic enzymes by rigorous homogenization, osmotic shock, sonication, glass wool filtration, and centrifugation. Preparations were substantially free of cellular debris and fibrous contaminants, as determined by electron microscopy (Fig. 6:1). Isolated lipofuscin granules had the same ultrastructure as they had <u>in situ</u>, with the same diversities in size, shape, electron density, and relative proportions of 'matrix' and 'vacuoles'.

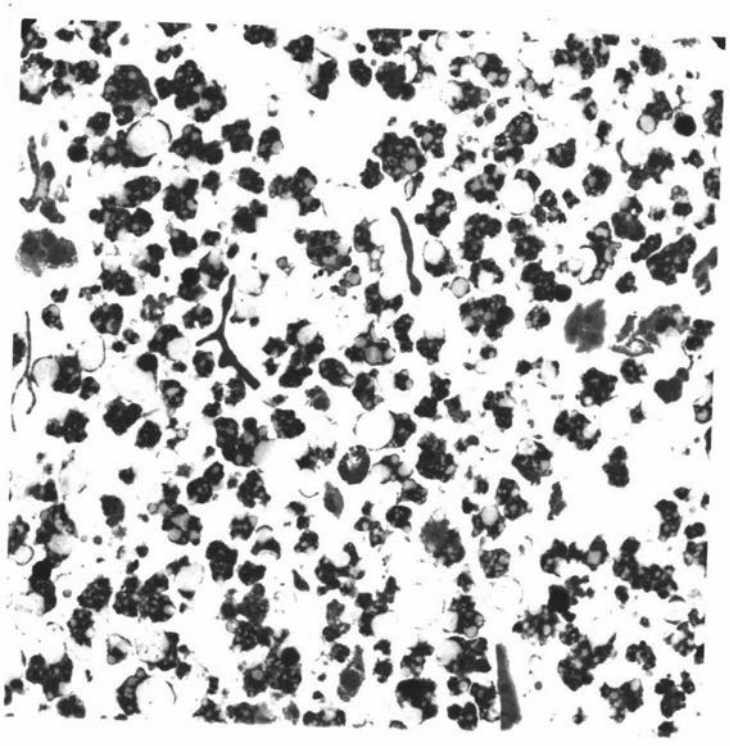


Figure 6:1.

Isolated lipofuscin granules. Preparations were substantially free of cellular debris and fibrous contaminants.

The purity of the isolate was not improved by the use of a sieve to homogenize thyroid tissue (Alquier et al 1985), or by the use of collagenase, with the attendant risk of introducing other exogenous proteases.

Isolates of lipofuscin were lyophilised, weighed, and expressed relative to dry thyroid weight. Lipofuscin yields varied considerably between individuals, but there was no correlation with age (Table 6:2).

Table 6:2. Yields of lipofuscin granules isolated from equine thyroid glands

Source age/sex	Weight wet tissue(g)	Weight dried lipofuscin (mg)	mg dried lipofuscin per g dried tissue
310/2s	31.91	16.1	2.0
309/4g	11.06	29.9	10.5
310/4g	17.10	36.7	8.4
480/9g	31.84	225.2	27.5
308/11f	27.90	74.7	10.4
523/12g	11.83	13.5	4.4
530/13f	16.18	12.4	3.0
531/14f	24.99	11.4	1.8
519/15g	19.34	8.8	1.0
531/15f	17.43	8.7	1.9
498/18f	27.96	37.0	5.2
512/18f	11.22	28.5	9.9
523/18g	9.33	13.0	5.4
530/18f	29.12	29.8	4.0
564/19g	9.59	33.0	13.4
307/20s	11.10	35.5	12.5
308/20g	5.61	35.9	24.9
311/20f	6.67	14.7	8.6
500/20f	37.51	31.2	3.2
509/20g	23.70	20.5	3.4
509/23f	12.65	29.8	9.2
531/24f	24.45	29.4	4.7
309/25g	16.03	73.8	17.9
531/28f	16.95	17.0	3.9
306/30g	8.18	22.2	10.6
312/30f	26.08	141.9	21.2
519/30g	8.67	19.5	8.7
523/30f	12.46	33.5	10.5
500/31g	7.06	19.3	10.7
312/32f	10.48	33.2	12.3
523/32f	9.49	23.5	9.6

Key: s = stallion g = gelding f = female

Density determination

Lipofuscin granules did not band to a single density on a 1 M to 3.4 M CsCl gradient, but were evenly distributed over a wide range of densities (Table 6:3). When samples were centrifuged for a much longer time, 56 hours, the same range of densities occurred indicating that isopycnic conditions had been reached.

Table 6:3. Density of lipofuscin granules

Horse Sex/age	Density range
f/17	1.14 - 1.41
g/20	1.17 - 1.34
f/21	1.12 - >1.47

Electron microscopic examination of samples of different density showed no obvious relationship between density and morphology although some loss of morphological detail occurred during the isopycnic centrifugation, probably due to the high salt concentration.

Elemental analysis of lipofuscin

Hydrogen, carbon, nitrogen, sulphur, and total halides were measured in samples of lipofuscin and thyroid tissue from four horses (Table 6:4). The proportions of these elements were similar between individuals. The nitrogen content of lipofuscin was consistent with the presence of proteins or nucleic acids, but was not as high as that of total thyroid tissue. In contrast, lipofuscin contained a higher proportion of halides. Carbon, hydrogen, and sulphur concentrations of lipofuscin were similar to those of thyroid tissue.

The concentrations of the metallic elements in isolated lipofuscin, and in thyroid tissue samples, from 11 horses were measured (Table 6:5). The mean metal content of thyroid tissue was 1.4% (Table 6:6). The contribution of metals to lipofuscin mass was highly variable, ranging from 0.9% to 4.4%, as was the concentration of individual elements.

Table 6:4. Elemental composition of equine thyroid lipofuscin: non-metallic elements (% by weight)

Horse	%H	%C	%N	%S	(%I)*	%P
4/gA	6.88	44.13	8.28	0.95	4.22	0.64
11/f	7.39	45.45	9.16	0.90	6.29	0.34
25/g	7.16	49.81	7.62	1.40	4.93	0.16
30/m	6.70	42.07	8.96	0.90	4.90	0.43
THYROID TISSUE						
mean	6.91	45.48	12.25	1.16	2.52	0.56
s.d.	0.10	0.48	0.37	0.15	0.65	0.18

*total halides, calculated as if it was all iodine

There was no age-related trend in the total amount of metal in lipofuscin, nor was there any age-related increase in the amount of any one metal in lipofuscin.

The concentration of each element in lipofuscin was compared to that in thyroid tissue from the same horse (Table 6:7). An element was considered to be 'stored' if the concentration in lipofuscin was twice, or more than twice, that in thyroid tissue. There was storage of most metals in lipofuscin. The stored metals were aluminium, calcium, chromium, copper, iron, manganese, molybdenum, nickel, strontium, and zinc. Lead was stored in four of the eleven animals. However potassium, magnesium, and sodium were not stored. Boron, not usually regarded as a metal, was stored. Phosphorus, another non-metallic element measured, was stored in only two animals.

Phosphorus accounted for 0.15% to 0.64% of the mass of the lipofuscin granules. If all the phosphorus present was a component of phospholipids, then the mass of phospholipid would be 25 x that of the phosphorus (Kates 1972), and would therefore account for a mean of 7.5% of lipofuscin mass (n = 11, s.d. = 3.7). However, some of the phosphorus in lipofuscin was a component of nucleic acids (see below) so actual phospholipid content must be below this figure.

Table 6:5. Elemental composition of equine thyroid lipofuscin: metallic elements, boron and phosphorus (ug/g)

Horse	Al	B	Ca	Cd	Cr	Cu	Fe	K	Mg	Mn	Mo	Na	Ni	P	Pb	Sr	Zn
2/s	349	41	3532	neg	13	17	383	294	337	2	neg	9299	neg	3814	neg	10	114
4/gA	186	15	2481	5	35	53	467	578	291	4	neg	15171	21	6391	23	9	171
4/gB	257	23	9311	neg	17	12	738	155	413	8	neg	2506	10	2139	neg	13	195
11/f	242	12	5611	4	5	119	285	216	408	5	2	2948	30	3357	neg	11	112
20/s	194	16	6372	5	8	35	839	303	607	10	5	3263	8	2475	14	10	383
20/g	150	15	9605	neg	10	21	4187	125	436	12	4	1673	8	1546	15	10	327
20/f	257	54	14077	neg	32	51	2984	227	503	34	3	4789	16	2893	neg	21	316
25/g	145	14	3410	neg	24	26	659	255	409	16	3	3858	14	1632	neg	7	212
30/g	878	176	14197	neg	44	42	1846	342	799	20	5	5315	10	2841	32	24	334
30/m	179	22	39347	neg	5	9	271	221	460	9	5	3667	5	4328	neg	21	179
32/m	170	20	7636	7	9	27	565	239	365	9	3	2905	4	1483	neg	10	296
Thyroid tissue																	
mean	20	18	1265	neg	neg	4	182	2803	492	2	0.3	9193	neg	5620	2	1	64
s.d.	28	28	814	-	-	2	146	1791	90	2	0.1	1451	-	1757	0.2	0.4	27

neg = negligible.

Table 6:6. Metal content of thyroid tissue and thyroid lipofuscin

Horse	Total metal/ dry tissue mg/g = %	Total metal/ dry lipofuscin mg/g = %	Lipofuscin metal/ dry tissue mg/g
2/s	10.7 = 1.1	12.7 = 1.3	0.02
4/g	10.3 = 1.0	19.5 = 2.0	0.22
4/g	13.2 = 1.3	13.6 = 1.4	0.11
11/f	11.4 = 1.1	10.0 = 1.0	0.10
20/s	15.8 = 1.6	12.1 = 1.2	0.17
20/g	17.3 = 1.7	16.6 = 1.7	0.42
20/f	15.4 = 1.5	23.3 = 2.3	0.22
25/g	11.3 = 1.1	9.1 = 0.9	0.18
30/g	16.6 = 1.7	23.9 = 2.4	0.29
30/f	16.6 = 1.7	44.4 = 4.4	0.52
32/f	15.7 = 1.6	12.2 = 1.2	0.23
	mean = 1.4% (s.d. = 0.3)	mean = 1.8% (s.d = 1.0)	mean = 0.23 mg/g (s.d. = 0.14)

Table 6:7 Ratio of stored* elements in lipofuscin compared to thyroid tissue

Horse	Element in lipofuscin/ element in tissue													Total %w/w of elements stored* in lipofuscin
	Al	B	Ca	Cr	Cu	Fe	Mn	Mo	Ni	P	Pb	Sr	Zn	
2/s	98	30	6	38	8	6	4	neg.	neg.	1	neg.	10	5	0.45
4/gA	15	2	4	86	12	4	3	neg.	48	1	11	10	4	1.86
4/gB	30	7	9	28	3	5	3	neg.	26	0.4	neg.	10	4	1.06
11/f	11	0.5	7	13	56	7	14	10	74	1	neg.	10	4	0.65
20/s	23	1	7	21	6	7	5	16	19	3	7	9	4	1.04
20/g	15	2	7	24	4	11	6	10	18	0.2	7	10	3	1.44
20/f	19	5	11	79	10	6	5	12	36	2	neg.	23	4	2.07
25/g	19	4	4	78	5	3	5	10	42	0.3	neg.	9	3	0.45
30/g	103	15	15	114	7	14	11	16	24	0.4	16	21	4	1.76
30/m	2	0.2	11	16	4	7	10	22	15	1	neg.	10	5	4.00
32/m	48	5	4	26	6	4	5	10	11	0.3	neg.	10	4	0.88

*an element was considered to be stored if there was a twofold or greater increase in ppm element in the lipofuscin compared to the tissue.

The proportions of different metals within lipofuscin isolates were highly variable between animals, with the exception of zinc. The ratio of lipofuscin zinc/thyroid zinc was consistently between 3 and 5 (inclusive).

Proteins

Solubility of lipofuscin and lipofuscin protein: None of the solvent systems tested completely dissolved lipofuscin proteins. Most of the lipofuscin was soluble in 98% formic acid, at a concentration of 1-2 mg lipofuscin/ml, when followed by 60 seconds sonication at full power. Less of the lipofuscin was soluble in 90% formic acid, as measured by the relative size of the insoluble pellet obtained after centrifugation. The addition of 2-mercaptoethanol did not affect solubility. Solutions of lipofuscin in 98% formic acid were not suitable for polyacrylamide gel electrophoresis, but were used for quantitative amino acid analyses.

Lipofuscin did not dissolve in the neutral detergents Triton X-100 or N-Tetradecyl-N,N-dimethyl-ammonio-3-propane sulphonate, nor in guanidine HCl. A large precipitate formed when the formic acid-soluble components were mixed with these detergents and the formic acid removed by neutralisation or evaporation.

To dissolve the protein in ionic detergent, for separation by LDS-PAGE, an aqueous solution of 6% LDS and 20% 2-mercaptoethanol was added to an equal volume of aqueous suspension of 1-2 mg/ml lipofuscin, followed by incubation for 5 minutes at 90^{o}C. Lipofuscin solubility was measured by comparison of quantitative amino acid analyses of the separated supernatant and pellet, and of a sample of the intact lipofuscin. The method dissolved 81% of the lipofuscin protein (range 79.3% to 82.5%, n = 3). Increasing the pH of the solvent solution with lithium hydroxide did not affect solubility.

Addition of a solution of 3% LDS and 10% 2-mercaptoethanol directly to a concentrated pellet of lipofuscin caused the formation of large clumps of lipofuscin and much poorer solubility as a result.

LDS-Polyacrylamide gel electrophoresis (LDS-PAGE): LDS-PAGE of lipofuscin proteins revealed that all samples (n = 7) had a group of four major protein bands in common, with apparent molecular weights between 14 and 18 kDa (Fig. 6:2). In some samples these were the major proteins present while in others numerous bands of higher molecular weight were also present. Greater sensitivity and superior resolution of the protein bands was obtained when gels were stained with silver, using the special silver stain described, compared to Coomassie Blue. The staining of the four major protein bands was greatly improved when the first silver stain was removed with Farmer's Reducer (see Chapter 3) and the gel overstained again with silver. In some cases the four major protein bands stained negatively, or were not apparent, with the first silver stain, but in all cases they stained well with the overstain. It was not possible to measure how much protein was loaded by the method of Lowry (1951), because the protein fraction was insoluble in the solutions used, and the addition of 2-mercaptoethanol interfered with the Lowry assay. A range of concentrations of each sample were loaded. Only a small proportion of the protein was retained in the stacking gel.

These four major protein bands extracted from isolated lipofuscin in chloroform/methanol/ammonium acetate (2:1:100 mM), remained in the chloroform layer when water was added, but precipitated upon the addition of ether. Proteins which can be dissolved and precipitated by these methods have been termed proteolipids (Folch and Lees 1951). This property enabled the separation of the four proteins from most of the other proteins in lipofuscin. This was confirmed by LDS-PAGE of the precipitate after addition of ether (Fig. 6:2), on which the proteins showed a lack of definition. The four proteins had similar amino acid composition to the total lipofuscin protein and total thyroid homogenates (Table 6:8). The lack of definition on LDS-PAGE following precipitation made electroblotting of the proteins difficult, so electroblotting of the total lipofuscin protein fraction was used to obtain samples suitable for amino acid sequencing. When electroblotted onto PVDF membrane the four proteins stained with Coomassie Blue and Amido Black stains (Fig. 6:2). They did not

Figure 6:2

Montage of lipofuscin proteins separated by LDS-polyacrylamide electrophoresis.

Lanes A to F stained and overstained with silver.

Lane A: Low molecular weight standards.

Lane B: Pellet of thyroid homogenate from 5 year old horse.

Lane C: Equine thyroid lipofuscin proteins pooled from a 25 year old mare and a 30 year old gelding.

Lane D: Thyroid total homogenate from an 18 month old filly.

Lane E: Equine thyroid lipofuscin proteolipids separated in chloroform/methanol/ammonium acetate.

Lane F: Non-proteolipid protein fraction from the same isolate as Lane E.

Lane G: Equine thyroid lipofuscin proteins electroblotted onto PVDF membrane and stained with Amido Black.

Four protein bands with molecular weights between 14 and 18 kDa (lanes C and G) are common to all lipofuscin isolates. These proteins are proteolipids (lane E), as they are largely removed by chloroform/methanol/ammonium acetate (lane F). Proteins which co-migrate with the proteolipids are detected in the pellet of the total thyroid homogenate of a 5 year old horse (lane B) but only a 14 kDa band is present in the total thyroid homogenate of an 18 month old filly.

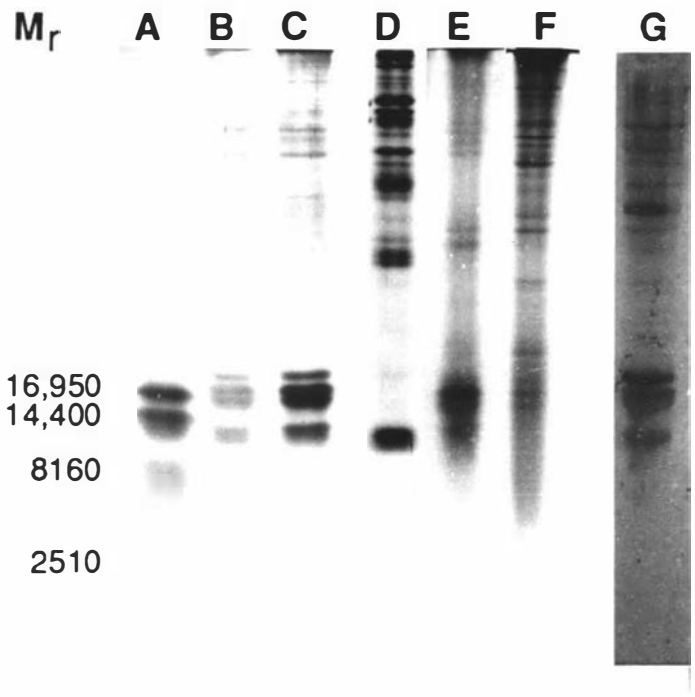

Mr
A
B
C
D
E
F
G
16,950
14,400
8160
2510

Table 6:8 Amino acid composition of thyroid tissue, isolated lipofuscin, and extracted proteolipid compared to thyroglobulins

Amino Acid	Mean molar% thyroid (n = 4)	Mean molar% lipofuscin (n = 25)[1]	Molar% proteolipid (n = 1)	Molar% bovine TG[2]	Molar% human TG[3]
Asp+Asn	8.6(+/- 0.6)	9.1 (+/- 1.1)	11.8	7.0	7.5
Thr	4.7(+/- 0.1)	4.8 (+/- 0.4)	4.2	4.6	5.3
Ser	6.8(+/- 0.5)	6.9 (+/- 0.8)	5.7	9.5	9.7
Glu+Gln	12.9(+/- 0.3)	10.3 (+/- 2.4)	13.6	12.4	13.1
Pro	7.3(+/- 0.5)	6.7 (+/- 0.5)	4.8	7.0	6.2
Cys	n.m.	n.m.	n.m	4.4	4.4
Gly	12.0(+/- 0.9)	12.5 (+/- 1.9)	12.1	7.7	7.6
Ala	9.0(+/- 0.5)	9.0 (+/- 0.6)	10.1	8.7	7.1
Val	6.3(+/- 0.3)	6.7 (+/- 0.4)	8.1	6.0	5.9
Met	1.2(+/- 0.3)	1.0 (+/- 0.4)	n.d.	0.9	1.4
Ile	3.1(+/- 0.4)	3.3 (+/- 0.4)	4.5	2.7	3.0
Leu	9.4(+/- 0.2)	9.2 (+/- 1.0)	9.3	9.6	9.4
Tyr	2.3(+/- 0.3)	2.7 (+/- 0.2)	3.9	2.6	2.4
Phe	4.7(+/- 0.3)	4.8 (+/- 0.4)	4.7	5.1	5.1
His	1.5(+/- 0.2)	2.0 (+/- 0.4)	1.1	1.1	1.5
Lys	4.5(+/- 0.8)	4.7 (+/- 0.4)	6.2	2.7	3.3
Arg	5.8(+/- 0.3)	5.4 (+/- 0.5)	n.d.	6.5	5.5
Trp	n.m.	0.7 (+/- 0.2)	n.m.	1.4	1.6

Key and Footnotes:
n.d. = not detected
n.m. = not measured
[1] n = 25 for all amino acids except tryptophan, for which n = 5.
[2]Molar percentages of amino acids in bovine thyroglobulin, as calculated from primary amino acid sequence (Mercken et al 1985).
[3]Molar percentages of amino acids in human thyroglobulin, as calculated from primary amino acid sequence (Malthiery and Lissitzky, 1987).

label with Concanavalin A, but stained negatively compared to the matrix. The four 14-18 kDa protein bands were detected in the pellet fraction of total thyroid homogenate from a 5 year old gelding (Fig. 6:2), which would contain lipofuscin as well as other constituents of thyroid tissue which are insoluble in water. A band migrating at a similar rate to the 14 kDa band was present in total thyroid homogenate from an 18 month old filly (Fig. 6:2), and in the water-soluble (supernatant) fraction of total thyroid homogenate from a yearling colt, but no bands corresponding to the other three proteins of interest were observed in these samples.

Amino acid composition of lipofuscin, proteolipid fraction of lipofuscin protein, thyroid tissue, and thyroglobulin: The molar ratios of amino acids in lipofuscin were highly repeatable between assays of several samples from the same horse and between samples from different horses, as shown by standard deviations in Table 6:8. The amino acid composition of lipofuscin was similar to the collective amino acid composition of the four 14-18 kDa proteins purified by extraction with chloroform/methanol/ammonium acetate (2:1:100 mM) followed by ether precipitation (Table 6:8). They were also similar to those in lyophilised, pulverised equine thyroid tissue, and to those calculated from the primary amino acid sequences of bovine and human thyroglobulin (Table 6:8).

There was no evidence for the presence of pyridosine or furosine, typically products of nonenzymatic glycation of lysine-containing protein (Mauron 1981).

Because the protein fraction of lipofuscin was not entirely soluble in LDS/2-mercaptoethanol, 19% of the protein could not be observed by LDS-PAGE. The amino acid composition of this fraction was similar to that of the soluble fraction and that of total lipofuscin protein (Table 6:9) with the exception that it contained a greater proportion of glycine.

Table 6:9 Amino acid composition of lipofuscin protein, lipofuscin protein fraction soluble in LDS/2-mercaptoethanol, and lipofuscin protein fraction insoluble in LDS/2-mercaptoethanol

Amino acid	Mean molar% lipofuscin (n = 25)	Mean molar% soluble fraction (n = 2)	Mean molar% insoluble fraction (n =2)
Asp+Asn	9.1 (+/- 1.1)	8.10	7.79
Thr	4.8 (+/- 0.4)	5.83	4.51
Ser	6.9 (+/- 0.8)7	6.73	5.76
Glu+Gln	10.3 (+/- 2.4)	10.68	9.31
Pro	6.7 (+/- 0.5)	6.38	8.37
Cys	n.m.	n.m.	n.m.
Gly	12.5 (+/- 1.9)	10.16	20.99
Ala	9.0 (+/- 0.6)	9.61	10.58
Val	6.7 (+/- 0.4)	7.06	7.26
Met	1.0 (+/- 0.4)	0.06	0.37
Ile	3.3 (+/- 0.4)	4.63	2.82
Leu	9.2 (+/- 1.0)	10.01	8.59
Tyr	2.7 (+/- 0.2)	0.84	1.24
Phe	4.8 (+/- 0.4)	4.67	3.87
His	2.0 (+/- 0.4)	2.35	1.42
Lys	4.7 (+/- 0.4)	7.47	3.11
Arg	5.4 (+/- 0.5)	5.46	4.40
Trp	0.7 (+/- 0.2)	n.m.	n.m.

n.m. = not measured.

Protein sequencing: J.E Walker, J.M. Skehel, and I.M. Fearnley at the M.R.C. Laboratory of Molecular Biology, Hills Road, Cambridge, UK, attempted to sequence the four 14-18 kDa proteins from the PVDF membrane blots. The pieces of membrane containing the Coomassie blue-stained bands of interest were cut from the blot and loaded into an Applied Biosystems 470A sequencer, in the presence of only the Teflon seal, with neither polybrene nor glass fibre discs, as described by Fearnley et al (1990). A number of amino acids showed at each cycle but no sequence information could be determined.

Protein content of lipofuscin: The protein contents of lipofuscin isolates from 18 horses were measured by quantitative amino acid analyses of hydrolysates. When different samples of isolated lipofuscin from the same horse were hydrolysed individually, there was considerable variability in the proportion of protein present (Table 6:10). The mean protein content of thyroid lipofuscin was 36% w/w but the range was from 15 to 77%. The variation between duplicate samples was not due to weighing errors as all samples were dried to constant weight, within 0.01 mg, over silica gel. Altering the duration of hydrolysis, to ensure that complete hydrolysis was achieved, was investigated. Samples usually showed a 1-2% loss of total protein mass, with the amino acid ratios remaining the same, for each 24 hour increase in hydrolysis time to 72 hours. However, in some cases the protein mass calculated was higher after longer hydrolysis, while in others the protein content was much lower. It was therefore concluded that the variation in protein content was probably due to heterogeneity within the isolate.

To investigate the problem of poor repeatability further, lipofuscin isolates from a further six horses were dissolved as much as possible in 98% formic acid, sonicated for 60 seconds at full power, and agitated while duplicate or triplicate aliquots were taken. The formic acid was evaporated and the aliquots hydrolysed for 24 hours. Results of quantitative amino acid analyses of these samples showed much less variability between samples from the same horse (Table 6:11). The mean protein

Table 6:10. Protein content of lyophilised equine thyroid lipofuscin

	Horse sex/age	% by weight protein; analysis # 1	2	3
1	g/4	50	-	-
2	f/11	52	-	-
3	g/12	28	36	-
4	f/14	31	-	-
5	g/15	31	-	-
6	f/15	34	-	-
7	f/17*	56	-	-
8	f/18	15	27	-
9	f/18	46	-	-
10	g/18	28	18	-
11	g/18	22	22	24
12	g/19	16	-	-
13	g/20*	47	-	-
14	g/20	42	-	-
15	f/23	27	43	-
16	f/24	27	-	-
17	g/25	40	-	-
18	f/28	16	-	-
19	f/30	77	-	-
20	f/30	33	-	-
21	f/30	49	16	25
22	g/30+	21	44	29
23	g/31	42	-	-
24	f/32	44	35	-
25	f/35*	69	-	-

* assay did not include proline.
mean protein content = 36% w/w (s.d. = 15%, n = 36)

Table 6:11. Protein content of equine thyroid lipofuscin in 98% formic acid

	Horse	% by weight protein; analysis# 1	2	3	mean	s.d.
1	g/28	47.4	53	-	50.2	4
2	g/26	32.5	33	34	33.2	0.8
3	f/18	35.5	36.7	36.8	36.3	0.7
4	f/13	42.2	43.2	43.2	42.9	0.6
5	g/31	22.5	23.9	-	23.2	1
6	f/28	12.6	15.1	-	13.9	1.8

mean protein content = 33.3% (s.d. = 13.2%, n = 6)

content of 33.3% obtained by this method was very close to the mean protein content obtained for lipofuscin hydrolysed directly, and the range between horses was again large, from 12.6 to 53%. These results confirm that the variability between samples of lipofuscin from the same horse reflects heterogeneity within the isolate.

The mean protein content of total thyroid homogenate was 55.3% (s.d.=2.1, n=4).

Lipids

Extraction of lipids from isolated lipofuscin into chloroform:methanol, 2:1 (Folch et al 1957) containing 0.05% w/v butylated hydroxytoluene (BHT), did not greatly alter the ultrastructure of the granules. Some fine detail of the matrix was lost, but the contents of the 'vacuoles' in the granules were not removed (Fig. 6:3).

Small amounts of phospholipid were obtained by acetone precipitation from the total lipid extract, and were analysed by thin layer chromatography. Phosphatidylcholine and phosphatidylethanolamine were the major components along with small amounts of sphingomyelin and lysophosphatidylcholine (Fig. 6:4). Phosphatidylserine and phosphatidylinositol were not detected, but this may be because of the small amount of lipid extracted.

Although good spectra were obtained by ^{31}P nuclear magnetic resonance (NMR) spectroscopy (Sotirhos et al 1986) of samples of control phospholipids extracted from ovine liver, the quantities of phospholipid obtained from samples of equine thyroid lipofuscin were insufficient to produce measurable spectra.

Neutral lipids were characterised by co-migration of purified standards and neutral lipids of hen egg yolk. The major lipid components extracted from lipofuscin and detected by thin layer chromatography co-migrated with dolichol and cholesterol (Fig. 6:5). The relative amounts of these lipids were visibly different between extracts from different horses. Triglycerides were not detected.

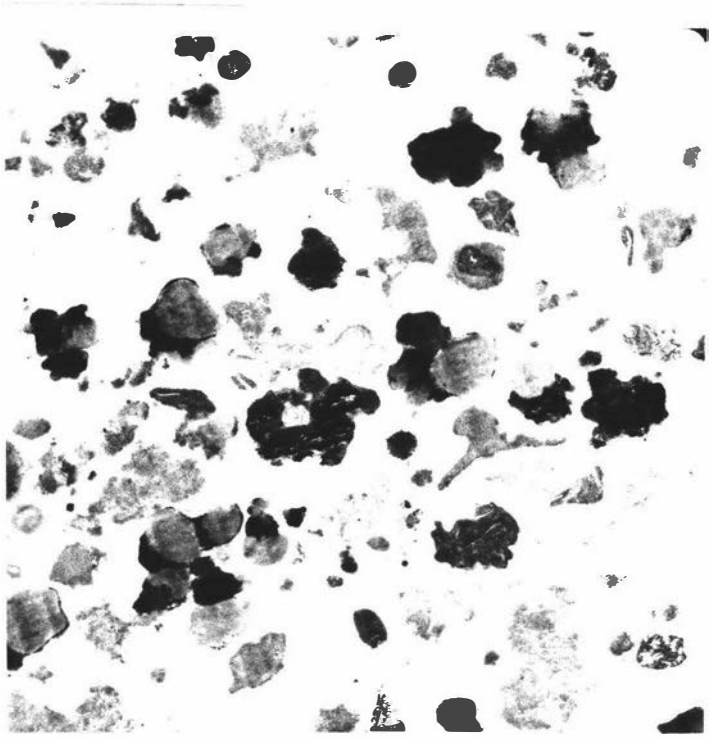


Figure 6:3

Lipofuscin granules after Folch extraction. The contents of the vacuoles are not removed from isolated granules by Folch extraction, and are therefore not lipid.

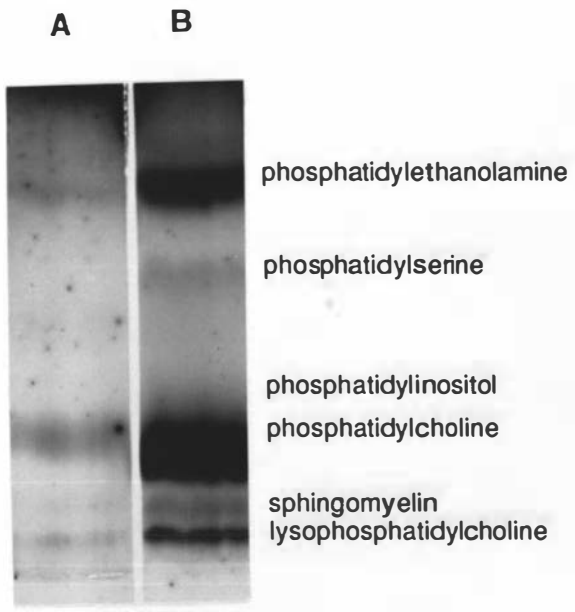


Figure 6:4

Thin layer chromatogram of phospholipids

Lane A: Lipids extracted from thyroid lipofuscin of 25 year old gelding.

Lane B: Lipids extracted from horse liver.

Small quantities of normal membrane phospholipids are present in equine thyroid lipofuscin.

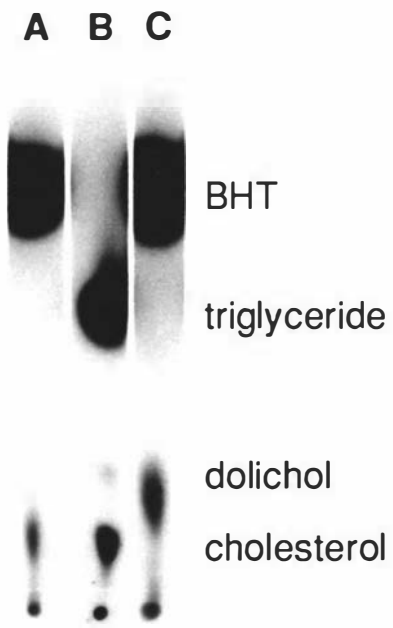


Figure 6:5

Thin layer chromatogram of neutral lipids.

Lane A: Neutral lipids of thyroid lipofuscin from an 18 year old mare.

Lane B: Neutral lipids from hen egg yolk.

Lane C: Neutral lipids of thyroid lipofuscin from a 24 year old mare.

Relative quantities of dolichol and cholesterol vary between horses. Triglycerides were not detected in any sample of neutral lipid from horse thyroid lipofuscin.

Quantitative analysis of dolichol and cholesterol by HPLC revealed great variation in concentrations in lipofuscin from different horses (Table 6:12). The variation was not age-related, and differences in dolichol and cholesterol concentration were independent of each other, but always much higher than the concentrations in total thyroid tissue. These two lipids accounted for 4-25% of the mass of lipofuscin.

Table 6:12. Neutral lipids of equine thyroid lipofuscin

Horse age/sex	Dolichol % by weight	Cholesterol % by weight
Thyroid homogenate		
5/gelding	0.003	0.17
Thyroid lipofuscin		
12/gelding	3.4	0.3
16/gelding	2.7	8.8
18/gelding	2.1	negligible
20/gelding	15.0	10.4
23/mare	15.0	2.7
25/gelding	7.0	19.2
30/mare	0.9	2.9
mean	6.6	6.3
s.d.	6.1	7.0

Carbohydrates

The strong positive staining of lipofuscin with Concanavalin A and PAS was indicative of the presence of carbohydrates, specifically mannose and/or glucose. Evidence for the presence of neutral sugars was also provided by a positive colour reaction with the phenol-sulphuric acid assay. Using mannose as a standard, neutral sugars represented an estimated 6.6 to 10.4% of the mass of lipofuscin (n=3). However the neutral sugar species present were unknown, and other chemical species which may react with the phenol-sulphuric acid assay were known to be present (see Discussion), so these data do not yield accurate estimates of the total sugar content.

Some hexosamines released from oligosaccharides by acid hydrolysis are detectable on ionic exchange amino acid analysis, although sugars

are more unstable than amino acids under conditions of acid hydrolysis. A small peak consistent with the retention time of glucosamine was occasionally detected in amino acid analyses of lipofuscin. To minimise destruction of hexosamine during hydrolysis, four samples of lipofuscin were hydrolysed for 6 hours and analysed by quantitative amino acid analysis with standards of glucosamine and galactosamine. Hexosamine was not demonstrated in lipofuscin by this method.

Peaks with elution times similar to those of glucose, mannose, galactose, myo-inositol, and glycerol were obtained by gas-liquid chromatography of hydrolysed lipofuscin. However, the repeatability of both qualitative and quantitative results between serial samples of lipofuscin was very poor. An internal standard of glucoheptose was largely, or completely destroyed during preparation of the samples.

Because of the unsatisfactory results obtained by such methods of direct carbohydrate analysis, the possible carbohydrate content was estimated indirectly by comparison of elemental and amino acid analyses. Elemental analyses of isolated lipofuscin from four horses were compared with quantitative amino acid analysis of the same four isolates. The hydrogen, oxygen, carbon, nitrogen, and sulphur which could be accounted for by the amino acids present were subtracted from the total quantities of those elements. The oxygen attributable to the mean levels of lipid and nucleic acid were also subtracted. The mass of metals measured was subtracted from the overall mass in each case.

The amino acids present accounted for 77% to 100% of the total nitrogen content and 29% to 78% of the total sulphur content. The mass of oxygen was calculated as the mass of the lipofuscin not accounted for by elemental analysis. The proportions of the carbon, hydrogen, and oxygen not accounted for by the proteins present were calculated (Table 6:13).

Table 6:13. Ratios of nonprotein carbon, hydrogen and oxygen in lipofuscin

Horse	Nonprotein C,H, & O %w/w of lipofuscin	Nonprotein Oxygen %w/w of lipofuscin	Oxygen as % of nonprotein fraction	Nonprotein C:H:O molar ratios
4/g	48.00	23.82	49.6	1.2: 1.9: 1
11/m	45.27	19.45	43.0	1.5: 3 : 1
25/g	55.94	20.24	36.2	2 : 3 : 1
30/m	26.30	16.70	63.5	0.6: 2 : 1
		-----	----	----------
	mean =	20.05	48.1	1.3: 2.5: 1

The nonprotein organic fraction calculated by this means in each isolate had a high oxygen content, consistent with the presence of carbohydrate. The C:H:O ratios in the nonprotein fractions were close to that of carbohydrate (empirical formula CH_2O). The mass of possible carbohydrate calculated from these elements accounted for 35.7% of the mass of lipofuscin, which was nearly all the mass not made up of protein, lipid, nucleic acid, or metal (see Discussion).

Nucleic acids

The mean DNA content by the Burton assay in isolated lipofuscin from nine horses, ranging in age from 7 to 31 years of age, was 2.4%, n=9, s.d. = 2.2, range =0 to 6%.

Nucleic acids extracted from thyroid lipofuscin isolated from a 7 year old mare without the use of sonication, were characterised by agarose gel electrophoresis in the presence of ethidium bromide (Fig. 6:6). The nucleic acid fraction consisted largely of DNA, as demonstrated by digestion with DNAse (Fig. 6:7). Most migrated as large molecules of DNA, with the same maximum molecular weight as DNA extracted from leucocytes from the same horse, although some DNA from both sources was fragmented. A small component of lower molecular weight was also present in the nucleic acid extracted from lipofuscin. This component stained with ethidium bromide, indicating that it was double-stranded, but was destroyed by incubation with RNAse and with DNAse, and was therefore identified as double-stranded heteropolymer (Fig. 6:7). DNA extracted from thyroid lipofuscin and the DNA from white blood cells from the same horse showed the same restriction enzyme digestion pattern (Fig. 6:8). Therefore the DNA in the lipofuscin was that of the horse, rather than viral DNA. The close similarity between leucocyte DNA and lipofuscin DNA indicates that the lipofuscin DNA is principally nuclear DNA, the most abundant DNA by mass in leucocytes, rather than mitochondrial DNA. The nucleic acid fraction of the thyroid lipofuscin from this horse, as measured by spectrophotometry, represented 5.6% of the dry weight of the lipofuscin.

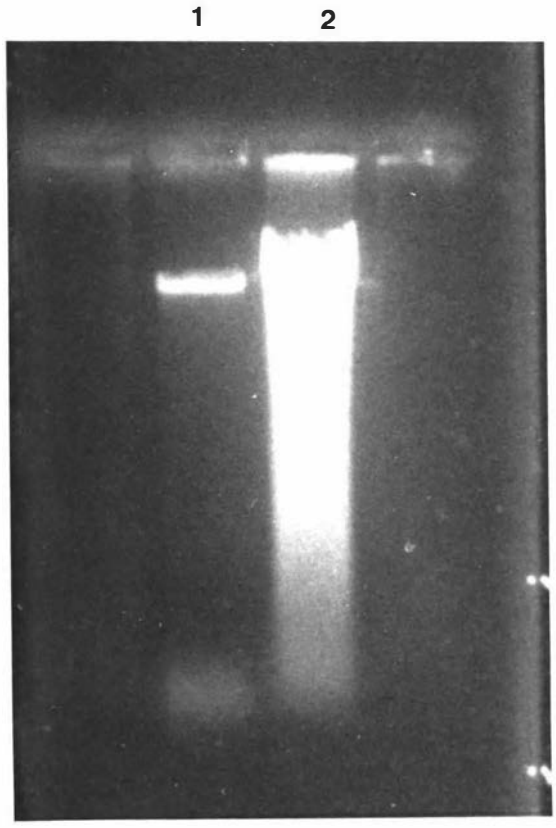


Figure 6:6

Agarose gel electrophoresis of nucleic acid from thyroid lipofuscin.

Lane 1: Lambda DNA (top) with slight contamination with RNAse-labile nucleic acid.

Lane 2: Nucleic acid from thyroid lipofuscin of a 16 year old mare.

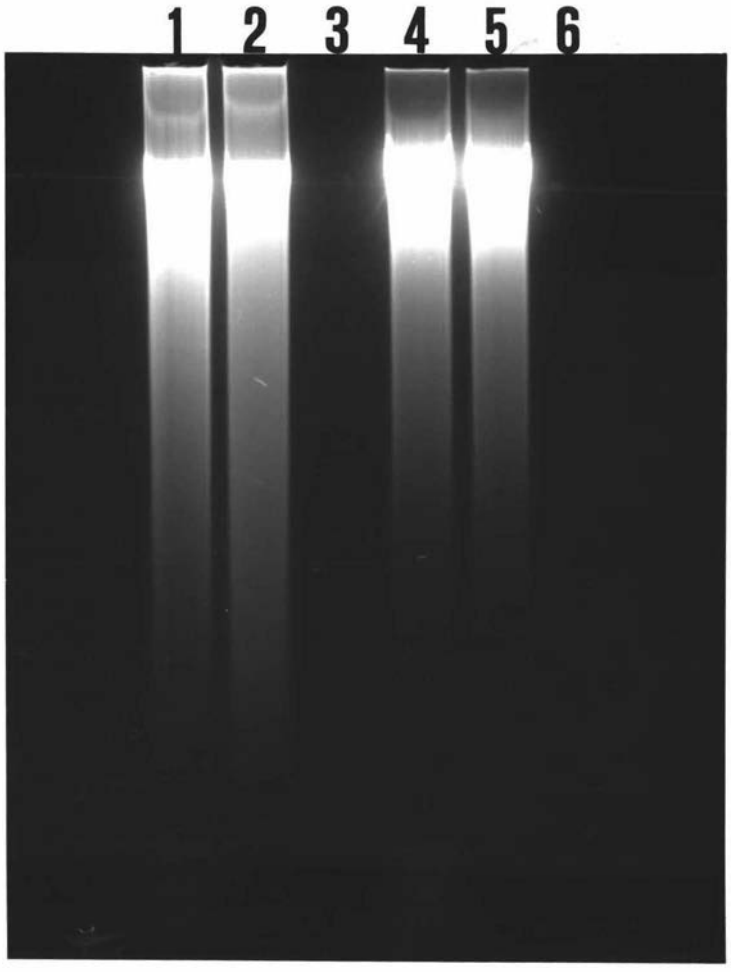


Figure 6:7

Agarose gel electrophoresis of nucleic acids after incubation with nucleases.

Lane 1: Nucleic acid from equine leucocytes.

Lane 2: Nucleic acid from equine leucocytes after incubation with RNAse.

Lane 3: Nucleic acid from equine leucocytes after incubation with DNAse.

Lane 4: Nucleic acid from equine lipofuscin.

Lane 5: Nucleic acid from equine lipofuscin after incubation with RNAse.

Lane 6: Nucleic acid from equine lipofuscin after incubation with DNAse.

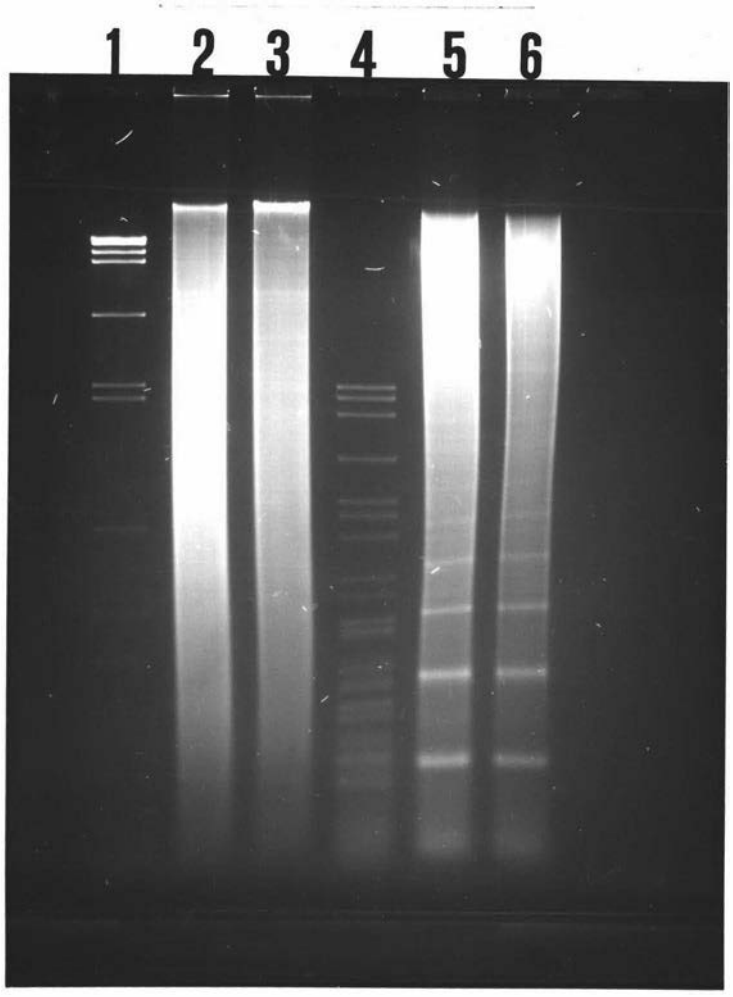


Figure 6:8

Agarose gel electrophoresis of nucleic acids after incubation with restriction enzymes.

Lane 1: Lambda DNA after incubation with DraI.

Lane 2: Equine leucocyte DNA after incubation with DraI.

Lane 3: Equine lipofuscin DNA after incubation with DraI.

Lane 4: Lambda DNA after incubation with MspI.

Lane 5: Equine leucocyte DNA after incubation with MspI.

Lane 6: Equine lipofuscin DNA after incubation with MspI.

DISCUSSION

The lack of correlation between the quantity of thyroid lipofuscin isolated and the age of the horse was consistent with the histological findings . Analysis of lipofuscin dolichol suggested that most of the lipofuscin in the thyroid was isolated by this method (see below).

Analysis of the various components of isolated lipofuscin revealed considerable heterogeneity in most of them, with great variability apparent between samples from different horses. This is consistent with the histochemical variability. However, in contrast to the variability in other components, the amino acid ratios were highly repeatable (standard deviations, Table 6:8), and four common protein bands, 14-18 kDa, were observed by LDS-PAGE in all samples thus examined. The discovery that these four bands behaved as proteolipids (Folch and Lees 1951) meant that they could be purified from other lipofuscin proteins by this property. Following this purification the proteins showed a lack of definition on LDS-PAGE. This is a characteristic of proteolipids (Fearnley et al 1990; Fig. 2). The great improvement in silver staining of the four proteins achieved when the stain was removed with a reducing agent and an overstain applied, was also observed in silver staining of the proteolipid stored in ovine ceroid-lipofuscinosis (Palmer et al 1986b, Fearnley et al 1990). No sequence information was obtained from the four proteins. They may be soluble in the solvents used in the washing cycles in the sequencer, and be washed out.
Alternatively they may be N-terminal blocked or have a ragged N-terminal. It cannot be inferred from the results that the four proteins are not acylated. The negative staining with Concanavalin A suggests that they are not glycosylated, but this reaction could be due to the physical nature of the proteins on the membrane.

The four proteins may be fragments of thyroglobulin, as suggested by the ultrastructural evidence (see Chapter 5). The amino acid ratios in isolated lipofuscin and in these proteins are very similar to those of equine thyroid total homogenate, in which thyroglobulin is

an abundant protein. Thyroglobulin makes up at least 3-5% of the wet weight of thyroid tissue (Wiersinga and Chopra 1982). Using the mean dry matter content measured in this study, thyroglobulin would therefore represent 12-20% of the dry weight of the gland, or 22-36% of the total protein content of thyroid, which is 55.3% of the dry tissue weight. Furthermore the amino acid ratios in lipofuscin and the purified proteolipids were also similar to those of human and bovine thyroglobulin (Table 6:8). The proteins in lipofuscin may be proteolytic intermediates of thyroglobulin. Lysosomal catabolism of thyroglobulin begins rapidly, with preferential release of the iodothyronines, but then becomes slower (van den Hove-Vandenbroucke 1980, Tokuyama et al 1987), and small peptides, accounting for 40 to 60% of the total protein, are produced as proteolytic intermediates (Peake et al 1970, van den Hove-Vandenbroucke 1980, Tokuyama et al 1987). This indicates that these proteolytic intermediates are more resistant to catabolism than the intact protein.

The proteolipid nature of the four 14-18 kDa proteins does not preclude the possibility that they are intermediates of thyroglobulin proteolysis. The term proteolipid does not mean that the molecules contain lipid or fatty acid moieties, or that they contain a high proportion of hydrophobic amino acids. Although relatively hydrophobic, the four 14-18 kDa proteins had a similar amino acid composition to thyroglobulin, a soluble protein. A hydrophobic nature can be the consequence of the tertiary structure of the protein rather than the amino acid composition. Proteins with a considerable proportion of polar amino acids are sometimes hydrophobic because the polar residues are on the inside and the hydrophobic residues are on the surface.

Alternatively, in light of the lysosomal nature of lipofuscin, it is possible that the four common proteins are lysosomal proteins, but this is unlikely because their molecular weights, in the range of 14-18 kDa, contrast with those of lysosomal enzymes. The subunits of the major enzyme by mass in porcine thyroid lysosomes, arylsulphatase A, have molecular weights of 58 and 54 kDa (Selmi et al 1989), while Yamamoto et al (1980) report that most lysosomal proteins have

molecular weights in the range of 60 - 110 kDa. If the proteins are derived from lysosomal proteins, they must be fragments rather than complete proteins. Another possibility is that the proteins are derived by autophagy from other organelles from the cell in which the lipofuscin granule forms, or that they are endocytosed fragments from other thyrocytes that have sloughed into the colloid.

The four 14-18 kDa protein bands were present in the pellet of total thyroid homogenate from a 5 year old horse, but this does not prove that the proteins are present in cellular components other than lipofuscin. Even at the relatively young age of 5 years, lipofuscin proteins may be a major fraction of the proteins in thyroid homogenate that are insoluble in water but soluble in a solution of LDS/2-mercaptoethanol. The group of four proteins was not found in the total thyroid homogenate of an 18 month old filly, although a band co-migrating with the 14 kDa band was present. It is therefore most likely that at least three of the four bands are not ubiquitous in the gland but are characteristic of the lysosomal system, perhaps of lipofuscin. The 14 kDa band may be a protein which is present in other cellular components. However this molecular weight is close to the limit of the resolution of the gel, so bands co-migrating at this apparent molecular weight may not be comparable with each other.

The protein solubility in detergent, 81%, was reasonable for an isolate of subcellular organelles. Because a proportion of such an isolate is commonly insoluble, it is normal practice that such samples in LDS/2-mercaptoethanol are centrifuged before gel electrophoresis.

The quantity of lipofuscin isolated from equine thyroid ranged widely, but there was no correlation with age. This result is consistent with that obtained by counts of lipofuscin granules *in situ* in a separate population of horses (see Chapter 4). Notably, the highest yield was obtained from a horse which was only 9 years old, and this yield was substantially higher than most yields from horses in their third or fourth decade.

Repeatable measurements of protein content were obtained by first dissolving the lipofuscin in formic acid, and dividing it into aliquots as a liquid. This method produced a very similar mean protein content as obtained by direct hydrolysis, which indicates that the heterogeneity in protein content between dry samples from the same isolate was genuine, rather than a result of experimental error.

The high densities of granules are incompatible with a large lipid component. This is in contrast to reports that lipofuscin is primarily composed of lipid or lipid peroxide products (Dolman and Macleod 1981, Nakano et al 1989). The considerable variation in density within isolates is likely to be due to heterogeneity between granules in the quantities of chemical species present. There was no evidence that the variation in density was due to the relative proportions of matrix and 'vacuoles'. Although the 'vacuoles' have been termed 'lipid vacuoles' or 'lipid droplets' by many authors, their contents were not removed by Folch extraction and are therefore not lipid.

Lipids invariably accounted for much less than half the mass of equine thyroid lipofuscin. No triglycerides were observed on TLC of lipids from six horses and no unidentified lipids were detected. Only small amounts of phospholipids were extracted, and these were the normal major membrane components: phosphatidylcholine, phosphatidylethanolamine, and small amounts of sphingomyelin and lysophosphatidylcholine, in the proportions typical of membranes. Based on the phosphorus content, phospholipids could account for 7.5%, on average, of lipofuscin mass. However, since nucleic acids are often present in small amounts, and phosphorus accounts for approximately 10.4% of the mass of nucleic acids, the phospholipid mass calculated from the phosphorus content is likely to be a considerable overestimate. The mean DNA content of lipofuscin was 2.4%, so the phosphorus in DNA would represent, on average, 0.25% of the mass of lipofuscin. When this is subtracted from the mean phosphorus content of lipofuscin, 0.30%, it allows a mean phospholipid content of only 1.25% w/w. However, several lipofuscin

samples contained no detectable DNA, and the actual phospholipid content may be highly variable, like the neutral lipid content.

In contrast to the phospholipids the neutral lipids were present in large proportions in some individuals. The dolichol concentration in isolated equine thyroid lipofuscin was extremely high relative to that of equine thyroid homogenate (Table 6:12). The high dolichol content of equine thyroid lipofuscin is consistent with a lysosomal origin, as free dolichol is largely located in lysosomes, and active storage of dolichol in lysosomes has been proposed (Wong et al 1982). However the quantity of dolichol in rat liver lysosomes, 15.11 ug/mg protein (Wong et al 1982), is very much lower than that found in lipofuscin in this study (Table 6:12).

Relatively high levels of dolichol are found in glands such as human thyroid, testis, adrenals, pituitary, (Rip et al 1985, Tollbom and Dallner 1986), liver (Rip et al 1985), and pancreas (Tollbom and Dallner 1986). The mean dolichol content of thyroid tissue from humans aged 23 to 95 years was 1145 ug/g, or 0.1% w/w (Rip et al 1985) while that of thyroid tissue from humans aged 68 to 74 years was 1960 ug/g (Tollbom and Dallner 1986), approximately twice as much. Free dolichol levels increase with aging in a range of tissues from humans and experimental rodents (Ng Ying Kin et al 1983, Pullarkat et al 1984), possibly as a consequence of a lack of a catabolic pathway (Rip et al 1985). Since the lipofuscin in the 23 year old mare represented approximately 0.9% of the tissue mass (Table 6:2), the lipofuscin dolichol (Table 6:12) alone would have given a total thyroid dolichol level of 0.14%, a level comparable to those reported for human total thyroid tissue homogenate. This supports the conclusion that most of the lipofuscin in the equine thyroid tissue was isolated. It also indicates that lipofuscin is probably a major storage site of dolichol in equine thyroid. It is possible that the dolichol measured in thyroid tissue of other species may be also largely located in lipofuscin granules. However, there is no evidence that dolichol is evenly distributed between lipofuscin granules in an individual. It is possible that dolichol is found only in a subpopulation of granules.

Equine thyroid lipofuscin contains a wide range of metals, that have diverse sources, biological roles, relative toxicities, and biological half-times (Friberg et al 1979). Some of these metals have been demonstrated by energy-dispersive x-ray analysis in thyroid lipofuscin in other species (Gordon et al 1984). In the horse, the concentrations of the important biological electrolytes, potassium and magnesium, were not higher in lipofuscin than in thyroid tissue, while that of sodium was only slightly higher. The concentrations of other metals were generally higher in isolated lipofuscin than in thyroid tissue, but there was no age-related trend in storage of any metal, and the metals varied independently of each other. The concentration of zinc in lipofuscin was consistently three to five times that in thyroid tissue. The wide range of concentrations of the various metallic elements in lipofuscin is probably a reflection of the exposure of the horses to each element. However, since the overall exposure to metals should increase with age, the lack of an age-related increase in the metal content of lipofuscin suggests that some turnover or excretion of the metals exists. An excretion pathway for entire lipofuscin granules may also exist, as discussed in Chapter 4.

The results suggest that no one metal is essential to the formation of equine thyroid lipofuscin, with the possible exception of zinc, the storage of which is consistent. The iron in lipofuscin may originate in part from microhaemorrhages of erythrocytes into follicular lumina occasionally observed in sections from horses in this study. Thyrocytes have been shown to endocytose erythrocytes from the colloid in vivo (Wollman 1980). A positive reaction to Perl's stain for iron was confined to a subpopulation of lipofuscin granules in paraffin sections. Microhaemorrhage may have occurred only in the follicles in which those granules were observed. The other metals found in equine thyroid lipofuscin may likewise be restricted to subpopulations of granules.

The mass of the halide fraction found by elemental analysis of lipofuscin and total thyroid tissue was calculated as iodide,

although some chloride was undoubtedly present. Lipofuscin contained approximately twice as much halide as total thyroid tissue. Concentrations of iodide higher than those of the surrounding cytoplasm have been previously demonstrated by analytical ion microscopy in murine thyroid lipofuscin (Mestdagh et al 1990) and by energy-dispersive x-ray analysis in canine, cavian, simian, and human thyroid lipofuscin (Gordon et al 1984). The presence of iodide in lipofuscin is not surprising, given the role of lysosomes in the catabolism of thyroglobulin and liberation of T3 and T4 (Tice 1977, Wollman 1980, van den Hove-Vandenbroucke 1980), and the evidence in this study that lipofuscin may contain fragments of endocytosed thyroglobulin. Mestdagh et al (1990) showed, by the use of ^{125}iodine tracer, that the iodide in lipofuscin has a slower turnover rate than that in the colloid, and suggested that this iodide may be excluded from hormone synthesis. However although the ages of the horses in which lipofuscin halide was measured ranged from 4 years to 30 years, the halide content of the lipofuscin did not increase but was similar in all four horses (Table 6:4). This suggests that although the turnover rate of iodide in lipofuscin may be slow relative to that of iodide elsewhere in the thyroid, it occurs at a steady rate.

The DNA in equine thyroid lipofuscin is variable in quantity. It shows the same maximum molecular weight and restriction endonuclease digestion as leucocyte DNA from the same horse. The great proportion of DNA in leucocytes is located in cell nuclei rather than mitochondria, and is of much higher molecular weight, therefore the lipofuscin DNA is also largely of nuclear origin. The presence of DNA within lipofuscin granules is consistent with the histochemical evidence (see Chapter 5), and therefore the DNA is unlikely to be a contaminant. This raises the question of how nuclear DNA comes to be found in a cytoplasmic organelle. It is likely that the DNA originates from the nuclei of the cells occasionally observed, usually degenerating or dead, in the follicular lumina. The autolysed contents of these cells, including their nucleic acids, may be endocytosed by the active thyrocytes of the follicle and thus the DNA enters the lysosomes of those cells. However, gel electrophoresis showed that the DNA was not greatly degraded as might

be expected if it had come from an autolysed cell. It was not more fragmented than DNA from fresh, viable leucocytes. The lipofuscin DNA may be DNA of the cell in which the lipofuscin is found. It is generally believed that DNA is broken down very slowly, if at all, in cells during life, but this makes it hard to explain why nucleases are abundant in many tissues (Davidson 1972). Given the importance of the control and turnover of DNA and RNA, it is interesting that very little is known about the control of nucleic acid catabolism.

The histological evidence for RNA was inconclusive (see Chapter 5). Although the nonprotein nitrogen content of 0-1.7% (n =4) theoretically allowed for a nucleic acid content of up to 10% w/w, the mean DNA content of 2.4% accounted for almost all of the mean phosphorus content of lipofuscin (see above). The remaining phosphorus would allow for a mean RNA content of only 0.5%, and then only provided no phospholipid was present. It is therefore most unlikely that RNA is a significant component of the mass of equine thyroid lipofuscin.

The remainder of the lipofuscin proved difficult to analyse, but circumstantial evidence indicates that it is mainly carbohydrate. Equine thyroid lipofuscin labelled with Concanavalin A in histological section, indicating the presence of glucose and/or mannose residues (see Chapter 5). The positive reaction of lipofuscin with PAS stain was most likely to be due to the presence of carbohydrate.

The ratios of nonprotein carbon, hydrogen, and oxygen (Table 6:13) closely approximated the C:H:O ratio of 1:2:1 found in carbohydrate, which is 53% oxygen by mass. The nonprotein fraction of lipofuscin contained, on average, slightly less oxygen than this (Table 6:13). The other nonprotein organic molecules found in lipofuscin, lipids, and nucleic acids, cannot account for the oxygen content of the nonprotein fraction. Equine thyroid lipofuscin contains, on average:

Protein	36.0%	w/w	(Table 6:10)
Dolichol	6.6%	w/w	(Table 6:12)
Cholesterol	6.3%	w/w	(Table 6:12)
Phospholipid	1.3%	w/w	(see above)
DNA	2.4%	w/w	(see above)
Metals	1.8%	w/w	(Table 6:6)
Iodide	5.1%	w/w	(Table 6:4)
	59.5%	w/w	

Oxygen represents 32% of the mass of DNA, but only 7% w/w of phospholipid, 1.14% w/w of dolichol, and 4.15% w/w of cholesterol. Therefore the mean values of these components, above, would account for a nonprotein oxygen content of:

Oxygen in dolichol	0.08% of total mass
Oxygen in cholesterol	0.26% of total mass
Oxygen in phospholipid	0.01% of total mass
Oxygen in DNA	0.77% of total mass
	1.12% of total mass

The nonprotein oxygen content is in fact much higher (Table 6:13), with a mean value of 20.05%. If the oxygen accounted for by the lipids and DNA is subtracted from this, oxygen representing 18.93% of the total lipofuscin mass remains in the unidentified fraction.

If this oxygen is present as 53% w/w of carbohydrate, the mass of the carbohydrate would be 35.72% of the total lipofuscin mass, and the composition of lipofuscin would be:

Protein	36.0%	w/w	(Table 6:10)
Dolichol	6.6%	w/w	(Table 6:12)
Cholesterol	6.3%	w/w	(Table 6:12)
Phospholipid	1.3%	w/w	(see above)
DNA	2.4%	w/w	(see above)
Metals	1.8%	w/w	(Table 6:6)
Iodide	5.1%	w/w	(Table 6:4)
Carbohydrate	35.7%	w/w	(calculated from oxygen)
	95.2%	w/w	

Carbohydrates in lipofuscin could be present as side-chains of a glycoprotein, an obvious candidate being thyroglobulin or fragments of it. Thyroglobulin contains 10% carbohydrate by weight, and the ultrastructural study and amino acid composition indicate that thyroglobulin probably contributes to thyroid lipofuscin. There are many other possible sources of carbohydrate in lipofuscin, including lysosomal glycoproteins. All lysosomal membrane proteins described to date are glycoproteins, and some have been found to be 50-70% carbohydrate, while the luminal proteins of primary lysosomes are typically about 10% carbohydrate (Storrie 1988).

Analysis of the carbohydrate content of lipofuscin was fraught with difficulties. The available methods of carbohydrate analysis are for use upon a purified sample of polysaccharide or glycoprotein. Attempts to obtain such a sample by borohydride reduction were not successful. This may have been due to the presence of other molecules which react with sugars under conditions such as heating. Analyses were frustrated because nothing was known of the form in which carbohydrate was present, or of the nature of any bonds between carbohydrates and other molecules. Direct colourimetric analysis using the phenol-sulphuric acid assay produced a strong colour reaction. However to obtain reliable quantitative results with the

phenol-sulphuric acid assay it is necessary to know the relative quantities of different neutral sugars present, as they do not all give as strong a reaction as mannose (Beeley 1985). If most of the reacting substances are neutral sugars other than mannose, the content of 6-10% calculated from this assay may be a considerable underestimate. On the other hand a number of other known components of equine thyroid lipofuscin give false positive reactions with this assay, namely deoxyribose (Beeley 1985), protein, and heavy metal ions (Chaplin 1986).

The insoluble complexes produced from nonenzymatic glycation of sugars with proteins (Maillard products) are yellow-brown in colour and fluorescent (Mauron 1981), and therefore offer an attractive explanation for these properties in lipofuscin. Pyridosine and furosine, the early glycation products of lysine, were not found in lipofuscin. The glycated amino acids and further derivatives are not recognized as their original forms by quantitative amino acid analysis, so if significant quantities of Maillard products are present in lipofuscin, there should be nitrogen in excess of that accounted for by the amino acids recognized and quantified. In fact there is little nitrogen that cannot be accounted for by these amino acids. The lack of nonprotein nitrogen also militates against the presence of Schiff bases, the alleged products of *in vivo* lipid peroxidation.

SUMMARY

The quantity of lipofuscin isolated from equine thyroid ranged widely, but there was no correlation with age.

There was considerable heterogeneity in chemical composition of equine thyroid lipofuscin between individual animals and, in the case of protein quantity, between samples from the same animal.

In contrast to this heterogeneity the amino acid ratios of the protein fraction were highly repeatable, and proportions were similar to those in thyroglobulin. The average protein content was 36%, of

which 81% could be dissolved in LDS/2-mercaptoethanol. Four protein bands between 14 and 18 kDa were observed by LDS-PAGE in all samples thus examined. These proteins were soluble in chloroform/methanol/ammonium acetate.

The major lipids present were dolichol and cholesterol, but the proportions differed between horses. Small quantities of normal phospholipids were extracted. No triglycerides were observed on TLC of lipids from six horses and no unidentified lipids were detected. Lipids represented a mean of 13.7% of the mass of lipofuscin. The contents of the vacuole-like components of lipofuscin granules were not removed by lipid solvents and are therefore most unlikely to contain lipid. This is consistent with the histochemistry.

The concentrations of most metals were generally high in isolated lipofuscin relative to thyroid tissue, but there was no age-related trend in storage of any metal. The metal content in equine thyroid lipofuscin is likely to reflect the exposure to those metals of the horse during its lifetime.

A small quantity of nuclear DNA is present in lipofuscin in some horses.

There was colourimetric evidence for the presence of carbohydrate in lipofuscin, but direct quantitative analysis was not achieved, due to the complex nature of the material being analysed. Elemental analysis of lipofuscin strongly supports the hypothesis that up to 36% of the unidentified fraction may be composed of carbohydrate.

The good agreement between total lipofuscin nitrogen and lipofuscin protein nitrogen precludes the presence of significant quantities of hexosamines, products of nonenzymatic glycation of proteins, or Schiff bases.

Lipofuscin may represent a storage site for iodide, but since the halide content of lipofuscin does not increase with age, any storage of iodide in lipofuscin is unlikely to be permanent.

CHAPTER 7

GENERAL DISCUSSION

Experimental

There is a longstanding assumption in the scientific literature that lipid peroxidation is involved in biogenesis of lipopigments (see Review chapter). This theory is largely built upon the alleged similarities in fluorescence characteristics between lipofuscin and lipid peroxidation products formed *in vitro* (Chio and Tappel 1969a, b) but these similarities have been questioned recently (Eldred 1987, Brizzee and Ordy 1981, Hammer and Braum 1988). The development of ceroid in animals which are fed diets which increase lipid peroxidation *in vitro* is cited as supporting evidence for this theory, although such experiments do not always produce the same results (Porta 1987). There is no direct evidence for the extrapolation of the lipid peroxidation theory to include the biogenesis of lipofuscin in normal cells. The lipid peroxidation theory has been shown to be untenable for a number of forms of ceroid-lipofuscinosis in man and animals (Palmer *et al* 1986a,b,1989,1990,1991, Martinus *et al* 1991, Jolly *et al* 1991).

Lipopigments are generally assumed to be resistant to chemical analysis. In theories of lipofuscin biogenesis, the granules are often alleged to contain the indigestible products of an irreversible reaction, or series of reactions (Brunk and Collins 1981, Dolman and Macleod 1981, Zs.-Nagy 1988, Nakano *et al* 1989). In paraffin sections the granules are resistant to organic solvents (Elleder 1981) and isolated granules from human heart have proved difficult to analyse (Hendley *et al* 1963b, Bjorkerud 1964).

In recent years it has been found that experimentally induced disturbances in protein metabolism (Sharma and Manocha 1977, Manocha and Sharma 1977), and in particular lysosomal proteolysis, result in the accumulation of a lipopigment in a variety of tissues and species (Ivy *et al* 1984, Ivy and Gurd 1988, Katz and Shanker 1989). Furthermore genetic defects in protein metabolism have been

implicated in two inherited diseases in which so-called lipopigments accumulate, Chediak-Higashi disease (Essner et al 1974) and Ceroid-lipofuscinosis (Palmer et al 1986b,1989,1990,1991, Fearnley et al 1990).

This study was undertaken to determine the nature of lipofuscin in normal equine thyrocytes by the systematic use of histology, histochemistry, and chemical analyses, to determine the relationship between lipofuscin and age in this tissue, and to explore the possible relationship between lipofuscin and protein metabolism. The thyroid gland was selected as a suitable tissue for study because the metabolism of thyroglobulin is the central process in thyroid function, and there is a reasonable body of knowledge concerning this process.

The histological study of 31 horses showed that fine lipopigment granules first appeared in normal follicles by the age of 3 years. The number of granules per cell increased between 3 and 5 years but did not increase with age thereafter. No age-related increase in size or colour was observed after the age of 6 years. Because this lipopigment does not accumulate with age, the term 'age pigment' is not strictly appropriate, but since there is no evidence that the lipopigment is associated with disease of lipid metabolism, the term 'ceroid' is also inappropriate. Furthermore, in the great majority of follicles there was no evidence that the lipopigment was the product of any abnormal or pathological process, although unusually large granules were found in association with histological abnormalities of the colloid in a small proportion of follicles in young horses. The ubiquitous occurrence of lipopigment in horses over 5 years of age indicates that it is probably a normal feature of equine thyrocytes in the adult animal. The lipopigment forms in normal cells and has the morphological and histochemical characteristics of lipofuscin, and 'lipofuscin' has been used throughout this thesis as a descriptive term without connotations of a particular pathogenesis or association with age.

The lack of a progressive increase with age is not consistent with an end-stage residual body containing indigestible cellular debris

because the cellular burden of such bodies should increase progressively with age, unless they are excreted or diluted by cell division. It is probable that equine thyroid lipofuscin is formed throughout life, but also catabolised and/or excreted at a more or less steady rate. Lipofuscin granules in the colloid may be released from either exfoliated thyrocytes or healthy thyrocytes, and lipofuscin-laden macrophages in the interstitial tissue may represent another route of removal, but both of these were so rarely observed that they are unlikely to be major routes by which lipofuscin is removed. Therefore catabolism may be the more significant route for the disposal of lipofuscin. It is extremely unlikely that lipofuscin ceases to form in the adult horses. There was ultrastructural evidence that lipofuscin granules in the thyroids of horses in their twenties and thirties were active organelles, fusing with colloid droplets, and this is consistent with their lysosomal nature. It is also extremely unlikely that the absence of an age-related increase in lipofuscin is due to dilution of the cellular burden during mitosis. The mitotic rate of thyrocytes is low, and the age-related accumulation of cold follicles in murine thyroids suggests that most of the thyrocytes survive for the lifetime of the mouse. Furthermore in most horses there was a linear relationship between cell numbers and granule numbers within an individual gland (Table 4:1), which indicated that the lipofuscin burden was generally uniform throughout a section, with the exceptions of the abnormal follicles in the young horses. This uniformity militates against significant dilution of lipofuscin by mitosis.

The association between the presence of colloid abnormalities and the presence of large lipofuscin granules in a small number of follicles in horses up to the age of 6 years suggested that disturbances in follicular function may cause an increase in lipofuscin accumulation. It was not possible to determine whether the primary abnormality was in the thyrocytes or the colloid. Abnormalities in the colloid occurred independently of large lipofuscin granules in thyrocytes, but large lipofuscin granules were found exclusively in follicles with abnormal colloid. The association between the two phenomena disappeared in older horses, although follicles containing colloid

abnormalities were found in almost every horse. The large lipofuscin granules were found in most of the young horses, and their absence in the older horses therefore probably indicates that they can be largely or completely catabolised. It is not clear why these granules cease to form in the older horses.

The majority of lipofuscin granules, considerably smaller than those in the abnormal follicles, were evenly distributed in normal follicles in unstained sections, and in Schmorl's-stained and Masson's Fontana-stained sections, from age 3 years upwards. In almost all horses over the age of 5 years, the granules were of sufficient size and colour to be detected in H&E-stained sections, although they were more readily observed by fluorescence microscopy or by the use of Schmorl's and Masson's Fontana stains.

There was histochemical evidence for the presence of iron, DNA, and mannose and/or glucose in lipofuscin granules. The high natural electron density suggested the presence of metals. The histochemical reaction with lipid stains was moderate and only rarely extended to the 'vacuoles' which in other studies have been assumed to be 'lipid droplets'. The contents of these areas were not soluble in lipid solvents, only small amounts of lipid were extracted from equine thyroid lipofuscin, and no triglycerides were detected, so these 'vacuoles' are not 'lipid droplets'. Since these areas are not empty, the term 'vacuole' is also strictly a misnomer.

It has been suggested that the biogenesis of age pigment begins with the 'vacuoles' (Samorajski and Ordy 1967). However small lipofuscin-like structures complete with both matrix and 'vacuoles', were observed by electron microscopy in colloid droplets (Fig. 5:17). These probably represent the earliest stages of lipofuscin granules, as suggested by Ives et al (1975). Although lipofuscin granules in various tissues have been shown to be lysosomal in nature (Dolman and Macleod 1981, Barden and Brizzee 1987, Goebel 1988, Clark et al 1989), there is debate in the literature over where the material initially forms (Dolman and Macleod 1981). The evidence in this study is that lipofuscin forms in phagolysosomes derived from colloid

droplets. There is no evidence that lipofuscin granules form outside the lysosomal system in the thyroid.

The variability in histochemical reactions indicated considerable chemical heterogeneity. Some histochemical reactions, such as PAS, Schmorl's and Masson's Fontana stains, were positive in all cases but varied in intensity between individuals, while others, such as Perl's stain for iron and Feulgen-NAH stains, were positive in a minority of granules in a section (Table 5:1). The chemical heterogeneity was confirmed by analytical studies of isolated lipofuscin. Lipofuscin granules proved relatively easy to isolate, although the quantity obtained from each horse was not adequate for a full range of analyses, even using microanalytical techniques, because of the quantities of sample required for accurate results.

There have been very few analytical studies on isolated lipopigments. A more thorough and comprehensive analysis was achieved here than in any other study of the composition of lipofuscin reported to date. A range of chemical species, accounting for most of the mass of the lipofuscin, were separated and analysed.

The mean protein content of lipofuscin was 36% by weight, but this varied greatly between horses. There was also variation in the protein content between different samples from the same horse (Table 6:10). In contrast the amino acid composition of the protein fraction was highly repeatable between horses. The amino acid composition was similar to that of total thyroid homogenate and those of human and bovine thyroglobulins. Approximately 81% of the protein fraction was soluble in 3% LDS/10% 2-mercaptoethanol. The proteins separated as discrete bands on polyacrylamide gel electrophoresis. Four major protein bands, MW 14 to 18 kDa, were found in all samples. In light of the known function of thyrocyte lysosomes, and the ultrastructural evidence that colloid droplets fuse with lipofuscin granules, it is likely that these proteins are fragments of thyroglobulin. It has been shown that the intermediate proteolytic products of thyroglobulin are less readily catabolised than the parent protein. Proteolysis of thyroglobulin begins rapidly but then

becomes slower (van den Hove-Vandenbroucke 1980) and 40 to 60% of the total degradation products of thyroglobulin accumulate as small peptides in secondary lysosomes (Peake et al 1970, van den Hove-Vandenbroucke 1980, Tokuyama et al 1987).

The four proteins extracted as proteolipids in chloroform/methanol/ammonium acetate, but their collective amino acid composition was similar to those of total lipofuscin protein, bovine thyroglobulin, and human thyroglobulin (Table 6:8). Their hydrophobic nature must therefore be due to their tertiary structures rather than a high concentration of hydrophobic amino acids, and hence does not preclude the possibility that they are derived from thyroglobulin. Sequencing of these proteins was attempted but no sequence information was obtained, so it was not possible to confirm their origin.

Equine thyroid lipofuscin showed only a slight to moderate reaction to lipid stains in frozen sections. Of lipids extracted from isolated lipofuscin, cholesterol, a normal membrane component, and dolichol, a component of lysosomal membranes, were the principal lipid species. The dolichol content was much higher than that usually found in lysosomal membranes (Wong et al 1982). Comparison with the dolichol content of total thyroid homogenate showed that lipofuscin represented a major storage site of dolichol in equine thyroid tissue (Table 6:12). The high levels of dolichol found in lipofuscin suggest active storage of this neutral lipid, which is believed to lack a catabolic pathway (Rip et al 1985). However, the considerable variability between horses in the quantities of dolichol and cholesterol in lipofuscin suggested that neither was vital to the biogenesis of the lipopigment. Only small quantities of normal membrane phospholipids were found in lipofuscin. There were no triglycerides or denatured lipids.

There was histochemical evidence for the presence of reducing sugars, and lectin histochemistry demonstrated the presence of mannose and/or glucose in equine thyroid lipofuscin. The elemental analysis indicated that up to 36% of the mass of lipofuscin is likely to be

carbohydrate. The normal approach is first to purify the glycoprotein, proteoglycan or glycolipid, then cleave off the carbohydrate, hydrolyse it into monosaccharides and quantify them. However attempts to purify carbohydrate for analysis were not successful, and attempts to analyse the carbohydrate without first purifying it were also unsuccessful. These results were not surprising given the diverse range of other chemical species in lipofuscin which would be likely to react with carbohydrates under conditions such as borohydride reduction or hydrolysis. No reliable techniques exist for analysing carbohydrates directly within samples of this nature. Only small quantities of nonprotein nitrogen were present, indicating that any carbohydrates present could not include large quantities of hexosamines or products of nonenzymatic glycation of proteins.

This is the first time that carbohydrate has been suggested as a major component of a lipopigment. Carbohydrate is the most likely explanation for the positive reaction of lipofuscin with PAS stain. In the past the positive staining with PAS has been attributed by a more convoluted argument to the presence of aldehydes produced by lipid peroxidation, apparently on the assumption that lipopigments do not contain carbohydrates. Carbohydrate may also be responsible for the reducing capacity demonstrated by the use of Schmorl's and Masson's Fontana stains. The carbohydrate in this lipofuscin could originate from thyroglobulin, which is about 10% of the molecule (De Nayer and Vassart 1980), or from other sources. Some lysosomal membrane proteins contain 50-70% carbohydrate, while lysosomal luminal proteins are typically about 10% carbohydrate (Storrie 1988).

A wide range of metals were found in equine thyroid lipofuscin (Table 6:5). There was no age-related increase in total metal content, suggesting that some recycling or excretion of the metals occurs in lipofuscin. The concentrations of metals were generally higher in isolated lipofuscin than in thyroid tissue, but the concentrations of the metals varied independently of each other, and independently of age.

Lipofuscin contained approximately twice as much halide as total thyroid tissue, a finding consistent with other studies of thyroid lipofuscin (Gordon et al 1984, Mestdagh et al 1990). The halide content of lipofuscin did not increase with age (Table 6:4), suggesting that this component, probably mostly iodide, is also recycled or excreted at a steady rate.

Equine thyroid lipofuscin and the lipid peroxidation theory

According to the lipid peroxidation theory lipofuscin consists of 'degenerated', 'degraded', 'crosslinked' material or 'polymerized residues of lipids and proteins' (Brunk and Collins 1981, Dolman and Macleod 1981, Ivy et al 1984, Zs.-Nagy 1988, Nakano et al 1989). Many findings in this study are incompatible with the lipid peroxidation theory of lipofuscin biogenesis and composition.

Equine thyroid lipofuscin has the typical colour, morphology, fluorescence and reducing capacity of lipofuscin, but it seldom stains with Oil Red O, and the moderate reaction to Sudan Black B is not greater in frozen sections than in paraffin sections, from which most lipids should be removed during processing. The simplest and most likely explanation for the positive PAS reaction is the presence of carbohydrates, as confirmed by lectin histochemistry.

The quantity of lipofuscin in the equine thyroid is not age-dependent, and intracellular turnover is the most likely explanation for this. Such catabolism is not compatible with the lipid peroxidation theory which proposes that lipofuscin is degraded, polymerised material resistant to catabolism (see Chapter 1).

No triglycerides or free fatty acids were found in equine thyroid lipofuscin, so the only possible sources of polyunsaturated fatty acids to be peroxidised are phospholipids, but only small amounts of normal phospholipids were found and the phosphorus content of isolated lipofuscin precludes the presence of significant quantities of 'degraded' or peroxidised phospholipids. Amino acids accounted for most of the nitrogen, and the small amounts left were likely to be in nucleic acids, so there cannot be significant quantities of Schiff bases.

Not all the protein was soluble in the detergent system used, but this does not mean that the insoluble protein is in any way 'degenerated', 'degraded', 'crosslinked' or 'polymerized'. Normal proteins may be insoluble in water and in solution of LDS/2-mercaptoethanol. Collagen is an obvious example of a normal protein which is insoluble in these and numerous other solvent systems.

The chemical heterogeneity of equine thyroid lipofuscin cannot be accounted for by a single cause, as proposed by the lipid peroxidation theory. The observed heterogeneity in morphology, histochemistry, and chemistry is likely to be a reflection of their lysosomal function. Most of the mass of this lipofuscin has been identified and there is no evidence of free radical-induced damage to the lipids or proteins present. There is no evidence for the presence of complex, polymerized material resistant to catabolism. If any such material is present, it does not represent a significant proportion of the mass of this lipofuscin.

Equine thyroid lipofuscin in paraffin sections was fluorescent, although not as intensely fluorescent as other structures in the same tissue, such as spherites and altered colloid. The latter are probably colloid from which some water has been lost. The basis for the fluorescence of the lipofuscin granules is unknown. There was no evidence that a specific fluorophore reflecting the biogenesis of the lipofuscin, as proposed by the lipid peroxidation theory, was present. It is likely that the fluorescence is a property of thyroglobulin fragments in that environment. The lipopigment which is stored in ovine ceroid-lipofuscinosis is intensely fluorescent in sections, but the isolated storage bodies dissolved in an aqueous solution of 1% LDS are not fluorescent, and negligible fluorescence can be demonstrated in chloroform/methanol extracts of the isolated storage bodies (Palmer and Westlake, unpublished data).

The results of this study indicate that equine thyroid lipofuscin granules are active lysosomal organelles, the accumulation of which is not directly related to age after maturity is reached. They are

ubiquitous in normal thyrocytes of adult horses, and are therefore probably normal lysosomal intermediates in these cells, although the association between unusually large lipofuscin granules and abnormalities of the colloid in young horses suggests that abnormal follicular function may sometimes increase the accumulation of lipofuscin.

Lysosomes, residual bodies, and lipofuscin

Lipofuscin granules have been described as lysosomal residual bodies (Barden and Brizzee 1987, Goebel 1988). This description is based on a model of lysosomal biogenesis and function which is now outdated. There have been changes in perception of lysosomes since their discovery by de Duve in the 1950s (de Duve 1969). In some early publications the lysosome was described as a cellular 'suicide bag', a concept now considered naive (Carroll 1989).

Lysosomes are heterogeneous in size and morphology (Carroll 1989, Kornfeld and Mellman 1989). They receive input from both endocytic and biosynthetic pathways, and share some biochemical characteristics with vesicles from both sources. Defining lysosomes by either morphological or biochemical criteria is therefore difficult. A useful functional definition of lysosomes is that they represent the terminal degradative compartment for macromolecules originating from within or outside the cell (Kornfeld and Mellman 1989).

The oldest theory of lysosome biogenesis is that each primary lysosome is produced from the Golgi complex as a complete organelle with a full complement of hydrolytic enzymes. Such a primary lysosome fuses with a vesicle containing substrate to become a secondary lysosome. Secondary lysosomes undergo multiple fusions with vesicles containing substrate. Eventually lysosomes become overloaded with insoluble material, and/or lose enzymic activity, and become defunct 'residual bodies' (Carroll 1989). However, this theory that primary lysosomes are created as fully formed units is unlikely to be valid, because there is evidence that newly synthesized lysosomal proteins are transferred with individual kinetics rather than a 'mass flow cohort' (Storrie 1988). For

example, a study of the transport kinetics of three animal lysosomal membrane proteins has shown that one protein was transported at a significantly slower rate than the other two (Barriocanal et al 1986).

The currently favoured hypothesis is that most lysosomal proteins are transported from the Golgi apparatus to the lysosome via vesicles which act as shuttles. Each shuttle does not carry a full complement of lysosomal proteins (Storrie 1988). These shuttles fuse either with an existing secondary lysosome (Storrie 1988) or with a late endosome carrying endocytosed material to the lysosome (Darnell et al 1986, Kornfeld and Mellman 1989). It is not established whether such endosomes then mature into lysosomes or whether their contents are transferred to lysosomes. According to the latter theory the late endosome represents a 'sorting station' from which mannose-6-phosphate receptors, plasma membranes, and Golgi membranes are removed and recycled, while lysosomal enzymes, lysosomal membrane proteins and macromolecules for degradation are directed into existing secondary lysosomes (Helenius et al 1983, Darnell et al 1986, Storrie 1988, Kornfeld and Mellman 1989).

The evidence in this study is that lipofuscin granules are active lysosomal organelles and are therefore likely to be serving some purpose, probably several purposes, in the cell. The same may be true of lipofuscin in other tissues, and other types of so-called 'residual body'. The model of the inert, defunct 'residual body' lysosome (Carroll 1989) is inaccurate, because it is now known that lysosomal enzymes are being continuously replaced with newly synthesized enzymes (Kornfeld and Mellman 1989).

Many, although not all, lysosomal proteins are directed to lysosomes by the presence of the lysosomal recognition marker mannose-6-phosphate (Darnell et al 1986, Storrie 1988, Kornfeld and Mellman 1989). A minority (5-10%) of lysosomal proteins with this marker are first secreted to the plasma membrane and recaptured by endocytosis (Kornfeld and Mellman 1989). Endocytosed thyroglobulin is also directed to the lysosomal system by the presence of the mannose-6-

phosphate marker (Herzog et al 1987). The lysosomes which fuse with newly endocytosed colloid droplets have been described as 'primary lysosomes' (van den Hove-Vandenbroucke 1980). However, in literature on the thyroid gland, the term 'primary lysosome' is sometimes used to describe recycling secondary lysosomes after thyroglobulin is catabolised (van den Hove-Vandenbroucke 1980, Selmi and Rousset 1988). This use of the term 'primary lysosome' is very different to that used by Carroll (1989) for a 'virgin' organelle.

The heterogeneity of lipofuscin is compatible with its lysosomal nature. There is strong evidence that many cell types contain heterogeneous subpopulations of lysosomes. Lysosomal heterogeneity in enzyme activity has been demonstrated in a number of studies, using tissues from a variety of experimental animals (Davies 1975), including rat hepatocytes (Groh and von Mayersbach 1981, Hultcrantz et al 1984) and rat spleen cells (Bowers and de Duve 1967). Thyroid lysosomes do not all contain the same range of lysosomal enzymes, as demonstrated by histochemical studies (van den Hove-Vandenbroucke 1980). Subpopulations of lysosomes which vary in metal content have also been identified in rat liver (Hultcrantz et al 1984) and spleen (Houghton et al 1971).

Some lysosomal heterogeneity, particularly in density, may be attributable to quantity and nature of the substrate contained in the lysosomes. For example thyroglobulin-enriched thyroid lysosomes have a lower specific gravity than thyroglobulin-depleted lysosomes from the same gland (Selmi and Rousset 1988). However, it is also likely that cells manufacture distinct subpopulations of lysosomes. Lysosomal enzymes turn over individually with half-lives ranging from less than a day to 65 days (Storrie 1988). The production of lysosomal enzymes at the endoplasmic reticulum or Golgi apparatus may also produce qualitative variations in the enzyme content of lysosomes (Davies 1975). Regardless of whether the heterogeneity of lipofuscin is due to different stages of catabolism or to manufactured differences in subpopulations of contributing lysosomes, it is still evidence that these organelles are functional rather than inert.

Possible mechanisms for biogenesis of equine thyroid lipofuscin

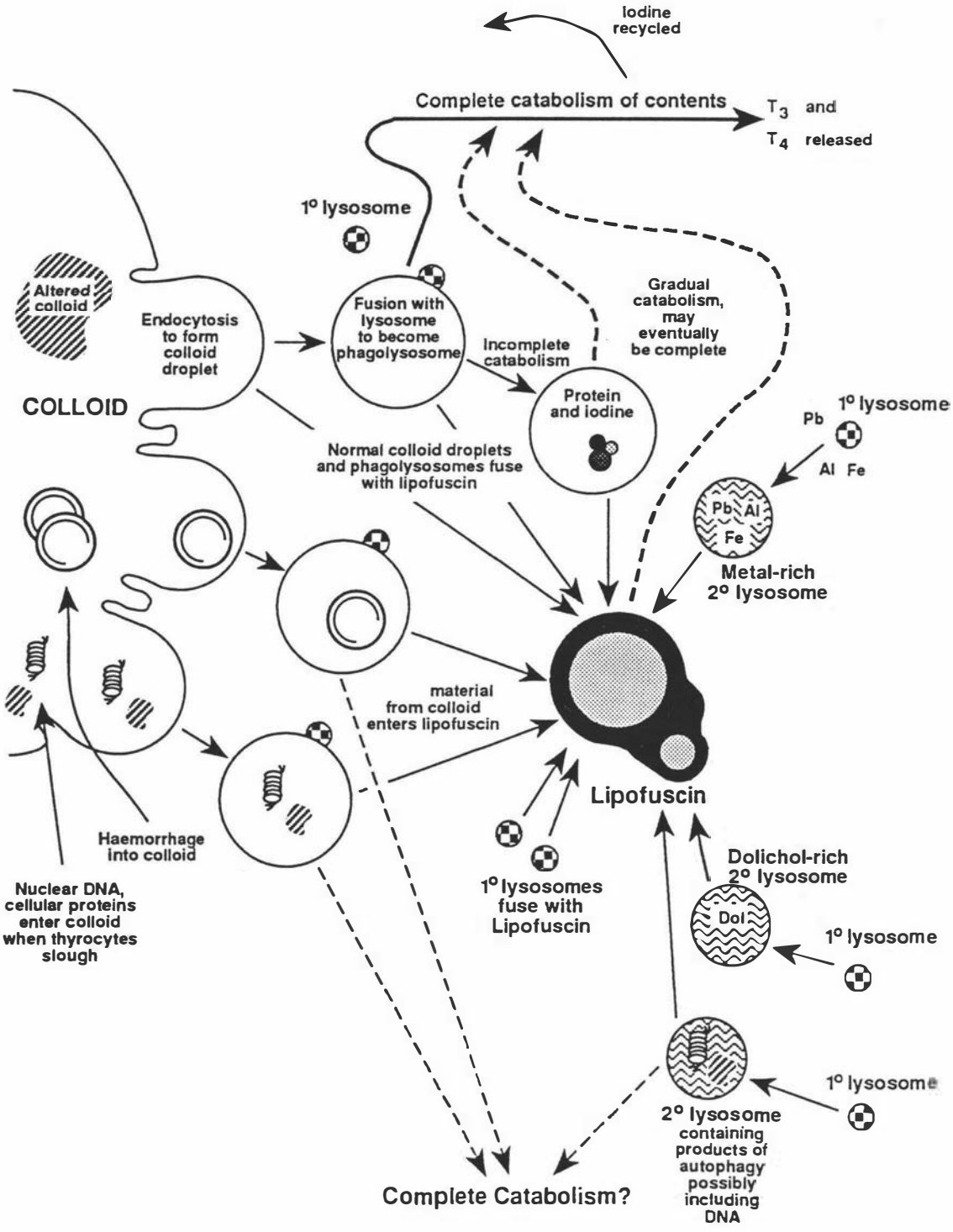
A proposed biogenesis, and possible pathways by which the various chemical species enter the lipofuscin granule, are illustrated in Fig. 7:1. For the sake of simplicity the traditional model of the 'primary lysosome' is used. At the top of the figure is the normal lysosomal pathway by which thyroglobulin is endocytosed and catabolised, with release of the thyroid hormones. In young horses, this catabolism may be particularly efficient due to a high metabolic rate. Alternatively, it may be no more efficient than that in mature horses, but accumulation of intermediates may occur at such a low level that they are not observed. In normal mature horses, catabolism is sufficiently slow that intermediates, recognized as lipofuscin granules, accumulate in phagolysosomes (derived from colloid droplets, see Fig. 5:17). The widespread presence of lipofuscin in thyrocytes of all horses of 5 years and over indicates that this is a phenomenon which occurs with maturation rather than with senescence. Fusion continues between lipofuscin granules and with colloid droplets which already contain some lysosomal enzymes. The protein, iodine, and carbohydrate in lipofuscin are probably derived from the thyroglobulin in the colloid droplets. Endogenous lysosomal glycoproteins are alternative sources of protein and carbohydrate (Storrie 1988).

If lipofuscin granules are an active part of the lysosomal system, then they are likely to serve some purpose in the thyrocyte. One possibility is that they help to smooth the release of thyroid hormones. For example, they might exert some control over the rate of recycling of iodide and tyrosine. This could help to explain the fact that although endogenous TSH is released in bursts (Hershman et al 1976, Condliffe and Weintraub 1979), when this is mimicked by experimental administration of a bolus of TSH, a steady state is reached during which the rate of thyroid hormone secretion is constant (van den Hove-Vandenbroucke 1980). However, if this is the case then highly active follicles should contain more lipofuscin granules than less active follicles, and no such relationship was discovered. A more likely possibility is that lipofuscin granules

Figure 7:1.

Proposed pathways of lipofuscin biogenesis.

Proposed pathways of lipofuscin biogenesis.



contain the peptide fragments which remain after the thyroid hormones are released from thyroglobulin. It is known that the thyroid hormones are preferentially released from thyroglobulin (van den Hove-Vandenbroucke 1980, Tokuyama et al 1987), that catabolism of thyroglobulin begins rapidly but then becomes slower (van den Hove-Vandenbroucke 1980), and that a substantial portion of the protein is not degraded to free amino acids but only to peptide fragments (Peake et al 1970, van den Hove-Vandenbroucke 1980, Tokuyama et al 1987). The assembly of an entire thyroglobulin molecule for the purpose of producing only two to four molecules of T_4 seems to be an unusually inefficient pathway (Tokuyama et al 1987). It has been suggested that the peptide fragments remaining after the release of the thyroid hormones may have some role in internal control of thyroid function, or possibly a hormonal role following secretion into the circulation (Tokuyama et al 1987). Whether or not these peptides have any physiological function in the thyroid gland, lipofuscin granules may serve as storage sites of the peptides and/or sites of their eventual catabolism.

However, if lipofuscin granules serve some useful purpose in thyroid metabolism, it is difficult to explain why they are rare or absent in immature horses. It is possible that they only become sufficiently large and/or long-lived to detect when other molecules from other sources also accumulate within them. These molecules may be catabolised more slowly, or stored for longer periods, than the products of thyroglobulin catabolism.

The proteins in thyroid lipofuscin have not been shown to be proteolytic intermediates of thyroglobulin. However, if the 14-18 kDa proteins are such intermediates, then they may be relatively resistant to catabolism because of their proteolipid nature. This property cannot be attributed to their amino aicd composition, and is most likely to be a consequence of their tertiary structure. If they are derived from thyroglobulin, then they may adopt this tertiary structure following the release of the iodothyronines from the parent protein.

The proteins and nucleic acids from thyrocytes exfoliated into the colloid may be endocytosed by surviving thyrocytes and enter the lipofuscin granules. Colloid which is altered in any way, for example by the loss of water, may also enter the lipofuscin granule but prove difficult to catabolise. Erythrocytes from microhaemorrhages into the colloid are known to be endocytosed (Wollman 1980) and may be the source of the high iron content indicated by a positive reaction to Perl's stain in some lipofuscin granules.

Endocytosis cannot, however, account for all the components in equine thyroid lipofuscin. It is unlikely to account for the neutral lipids, of which at least dolichol appears to be actively stored, or the great diversity of metals. Furthermore, at least some proteins, nucleic acids, and carbohydrates may be derived from autophagy. It is likely that other types of secondary lysosome, besides those formed from colloid droplets, also fuse with lipofuscin granules. Extensive exchange of membrane proteins and contents between lysosomes in the same cell has been demonstrated (Deng and Storrie 1988). In the thyrocyte, these may include secondary lysosomes containing products of autophagy, lysosomes which are rich in metals, and lysosomes in which accumulation of dolichol has occurred (Fig. 7:1). Variation in the proportion of the granule derived from each type of secondary lysosome (e.g. colloid droplet-derived phagolysosome, metal-rich lysosome) would give rise to the chemical heterogeneity found between granules isolated from the same individual, and in some cases found in the same follicle (Fig. 5:5, Fig. 5:9). However, not all secondary lysosomes necessarily fuse with lipofuscin granules. Their contents may be catabolised by other pathways (Fig. 7:1, dotted lines).

Ongoing catabolism or removal of most of the organic molecules, recycling of metals, and possibly recycling of dolichol, may also contribute to the chemical heterogeneity in granules. The rates at which various components enter or disappear from the granule may vary with time independently of each other, with the result that the relative proportions of different chemical species within a

lipofuscin granule may vary greatly over time. The total quantity of lipofuscin in the gland may also vary over time, although some granules are always present in adult glands.

The absence of an age-related increase in number of granules is in contrast to previous studies of thyroid lipofuscin. However, although both Matsubara et al (1982) and Ohaki et al (1986) concluded that there is an age-related increase in lipofuscin in human thyroid, in fact their results showed that in all age-brackets studied, some individuals had minimal lipofuscin in their thyrocytes. In both studies the apparent deposition of lipofuscin was studied by examining H&E stained sections and assigning one of five grades to each case. Grading of lipofuscin deposition was also used in the study of feline thyroids, although the granules were stained with the AFIP stain for lipofuscin (Ives et al 1975). The oldest cats in the study were 8 years old, and no increase in lipofuscin deposition was observed between 5 and 8 years (Ives et al 1975). Cats usually live into their teens, so 8 years is arguably only mid adulthood in the cat. It is possible that no age-related increase in lipofuscin quantity occurs in the feline thyroid after the age of 5 years. Examination of human and feline tissue by the method used in this study, using Schmorl's stained sections, counting both granules and cells in a selection of follicles and relating one to the other, rather than assigning a grade, might produce similar results to those found in the horse. It was found in this study that H&E stained sections can be deceptive, because the intensity of natural colour varies and granules can be obscured by the staining of the cytoplasm. However it is possible that an age-related increase in thyroid lipofuscin occurs in some species other than the horse. It may be that most or all horses die or are destroyed due to failure of other organs before the equine thyroid gland becomes senescent, while humans are maintained further into senescence. This appears impossible to prove or disprove.

Avenues of further study

The composition and heterogeneity of equine thyroid lipofuscin show that it is more complex in biogenesis and function than conventional

theories would suggest. From this general histochemical and analytical study, the proteins and carbohydrates seem to offer the most promising avenues for elucidating the role of lipofuscin granules in thyrocytes.

Further characterisation of the carbohydrate fraction is beyond the scope of this integrated general description. The qualitative analysis may be advanced by histochemistry using other lectins, but quantitative analysis of the carbohydrate without separation from the other components is not likely to be successful. Purification of the carbohydrate fraction is frustrated by the fact that, of the chemical species present, carbohydrates are the most likely to be destroyed by methods used to separate them from the other components. It is possible that nondestructive separation might be achieved by the use of exogenous lysosomal enzymes.

Other approaches to protein sequencing may permit identification of the four protein bands of interest. The use of antibodies raised against these isolated proteins might also prove fruitful.

The storage of proteolipids is a feature of this lipofuscin which is shared by the storage material in ovine ceroid-lipofuscinosis, which is histochemically similar to lipofuscin and which stains with lipid stains (Palmer *et al* 1989,1990,1991). The presence of these relatively hydrophobic proteins could be the reason why both these lipopigments stain with lipid stains. The possible presence of proteolipids has not been adequately explored in, or disproved by, studies of other lipofuscins in normal animals, and is another avenue for further study.

The equine thyrocyte could prove to be a suitable model for the experimental manipulation of lipofuscin formation *in vitro*. The endocrinology of the thyroid gland has been extensively studied, and methods are available for the *in vitro* culture of thyrocytes in thyroid slices and in reconstituted follicles, and the experimental manipulation and measurement of their activity. Tracer studies, either *in vivo* or *in vitro*, of radiolabelled amino acids and

components of nucleic acids, could be employed to elucidate the routes by which the proteins and nucleic acids enter equine thyroid lipofuscin. Tracer studies with radiolabelled iodine may also help to determine the role of endocytosed material in the biogenesis of thyroid lipofuscin.

As a model for the biogenesis of age pigment *in vivo*, the equine thyroid gland has the limitation that lipofuscin does not accumulate predictably with age. However a time-course study of lipofuscin accumulation in individual horses is not impossible *in vivo*, because thyroid tissue could be studied by the use of fine-needle aspiration biopsy, a technique widely used to diagnose the nature of thyroid nodules in humans.

It is very unlikely that lipofuscin in other tissue contains precisely the same chemical species as equine thyroid lipofuscin. The composition of lipofuscin probably varies from tissue to tissue depending on the predominant substrates for lysosomal catabolism and/or storage. Lipofuscin granules may be less active parts of the lysosomal system in other tissues. Age-related increases in lipofuscin have been reported in cerebral neurones and cardiac myocytes, both of which are highly specialized cell types which may not have the same capacity as thyrocytes to catabolise or perhaps excrete lipofuscin.

This study shows that extensive chemical analysis of lipofuscin is by no means impossible. Lipofuscins in other tissues may not be as resistant to chemical analysis as the literature suggests. Comprehensive chemical analysis of other lipofuscins must be attempted if their compositions and likely origins are to be determined.

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