

LIPOFUSCIN AND CEROID FORMATION: THE CELLULAR RECYCLING SYSTEM

Denham Harman

University of Nebraska
College of Medicine
Omaha, Nebraska 68105

SUMMARY

Lipofuscin, age pigment, is a dark pigment with a strong auto-fluorescence seen with increasing frequency with advancing age in the cytoplasm of postmitotic cells. By bright-field light microscopy lipofuscin appears as irregular yellow to brown granules ranging in size from 1-2mm in diameter. The fluorescent spectra of lipofuscin *in situ* generally show excitation maxima at about 360nm and a yellowish emission maxima at 540-650nm. Ultrastructurally the granules, localized in residual body-type lysosomes, are extremely heterogeneous and vary from one cell type to another, and frequently within a single cell. The pigment granules usually contain numerous liquid droplets embedded in an electron-dense matrix. The granules stain positively for neutral lipids but are not soluble in polar or non-polar lipid solvents. Lipofuscin contains about 50 percent by weight of proteinaceous substances, a lesser fraction of lipid-like material, and probably less than one percent by weight fluorophore(s); it is enriched in metals such as Al, Cu, and Fe, and in dolichols.

Free radical reactions and the proteolytic system are implicated in lipopigment formation. Thus the rate of lipopigment formation is increased by vitamin E deficiency and by increased intake of polyunsaturated fatty acids as well as by protease inhibitors such as leupeptin. Free radical reactions and proteolysis are involved in the continual turnover of cellular components. Cellular damage from free radical reactions, and others such as hydrolysis, has been present since the beginning of life. The evolution of more complex cells necessitated development of defenses - DNA repair processes, antioxidants, etc. - against damaging reactions as well as the removal and replacement of altered parts, and of those no longer needed by the cells. Proteins "marked" for disposal by oxidation damage, or other means such as conjugation with ubiquitin, are apparently rendered more hydrophobic so that they are "recognized" for degradation by the lysosomes and the proteinases and peptidases of the cytosol and mitochondria. Oxidatively altered lipids are removed by enzymes such as phospholipase A₂. The products of the degradation processes are reused by the cells.

Normally the recycling of damaged components works extremely well. There may be some slow slippage with advancing age as the rate of free

radical damage increases while protease activity decreases. As a result a gradually increasing fraction of lysosomal "food" may be converted to non-digestible forms, lipofuscin, before it can be broken down to reusable components. Ceroid is apparently formed when the disposal system is "overloaded" or impaired. Considering the complexity of this system there could be many genetic defects that result in ceroid formation in addition to those involved in Batten's disease.

KEY WORDS: Lipofuscin, ceroid, proteolysis, free radicals, evolution.

INTRODUCTION

Lipopigments can be categorized into those that occur normally, lipofuscin and neuromelanin, and the ceroids that are experimentally produced or associated with some pathological disorders. Lipofuscin was first observed in 1842, in dissected human neurons (Hannover, 1842), and neuromelanin in 1865 (Luys, 1865) in the human substantia nigra. Apparently ceroid was described first in 1936, in the uterus and kidneys of rats on a vitamin E free diet (Martin and Moore, 1936). Interest in lipofuscin was increased in 1866 when it was recognized that the pigment accumulated with age (Koneff, 1886). The voluminous literature that has accumulated on lipofuscin (Sohal, 1981; Totaro et al., 1987; Zs.-Nagy, 1988) is in part due to the hope that further knowledge of this lipopigment might contribute to an understanding of the aging process. Neuromelanin also accumulates with age; it is not as widely distributed as lipofuscin and is now considered to be melanized lipofuscin (Barden and Brizzee, 1987). Ceroid studies are prompted by the possibility that they will help in determining the mechanism of lipofuscin formation, for the two lipopigments have some properties in common, as well as by their presence in specific disorders. Included under the term, ceroid-lipofuscinoses are a group of autosomal recessive lysosomal storage diseases of humans and animals (Koppang, 1973/1974; Palmer, et al., 1986) associated with brain atrophy, blindness, dementia, and premature death. Human disorders include the Hermansky-Pudlak syndrome (Hermansky and Pudlak, 1959; Witkup et al., 1988) and the four subgroups of Batten's disease (Armstrong et al., 1982) infantile, late infantile, juvenile and adult (Kufs disease) (Berkovic, et al., 1988).

PROPERTIES OF LIPOPIGMENTS

Distribution

Lipofuscin has been found in the postmitotic cells of all species studied, from neurospora (Munkres, 1987) nematodes (Zuckerman, 1987; Epstein and Gershon, 1972) and flies (Miquel et al., 1977; Sohal, 1981) to humans (Brizzee and Ordy, 1981; Porta and Hartroft, 1969). The distribution in the human brain is uneven, being related to the level of oxidative enzymes (Friede, 1962, Friede et al., 1962), while it is uniform throughout the heart (Strehler et al., 1959).

Neuromelanin is not a "universal" pigment like lipofuscin. It is found only in mammals in the primates, carnivores, horse, and giraffe. Neuromelanin accumulates in several areas of the human brain stem, particularly the substantia nigra and the locus ceruleus as well as in the dorsal root ganglia of the spinal cord (Barden, 1981).

Ceroid can accumulate in both mitotic and post mitotic cells.

In situ Studies

Light Microscopy. Lipofuscin, neuromelanin, and the ceroids all appear about the same, as irregular yellow to brown cytoplasmic granules about 1-20 μm in diameter (Porta and Hartroft, 1969; Wolman, 1980; Barden, 1981). These pigments all display the same physical and biochemical characteristics at some time in their evolution (Porta and Hartroft, 1969; Miyagishi et al., 1967).

Electron Microscopy. The most prominent feature of the lipofuscin granules is their polymorphic internal configuration and the almost constant presence of a single membrane envelope (Porta and Hartroft, 1969). The granules contain varying numbers of: 1) translucent or almost translucent vacuoles, 2) compact, rounded or coarse bodies of variable electron opacity and osmophilia, usually very dense, and 3) laminated bodies of various shapes; they have been categorized into four groups on the basis of these characteristics (Miyagishi et al., 1967). The electron dense component of the granules increase with age (Siakotos et al., 1977).

The granules of ceroid are somewhat similar to those of lipofuscin. They accumulate in lysomes as rounded subgranules of 0.2 μm to several microns in diameter and often contain mixtures of small granular dense bodies, droplets of fat of moderate osmophilia, empty vacuoles or vacuoles with a peripheral rim of fat and sometimes laminated areas (Porta and Hartroft, 1969).

Histochemical. The lipopigments are relatively insoluble in polar or non-polar solvents. They stain positively for the presence of neutral lipids with oil red O, Sudan black or osmium. They are positive for polysaccharides with the periodic acid - Schiff reaction, are acid fast and exhibit moderate to strong basophilia. They are usually positive for acid phosphatase, a marker enzyme for lysomes (Oliver, 1981; Porta and Hartroft, 1969; 1981).

Autofluorescence. Autofluorescence is one of the most consistent features of the lipopigments; this property was first observed in 1911 (Stubel, 1911). The lipopigments exhibit a orange-yellow fluorescent when excited by near visible ultraviolet light, about 360nm (Wolman, 1980).

Isolated Lipopigments

Studies on the isolation and characterization of the lipopigments are few in comparison to the in situ ones, largely because the low concentrations of the lipopigments in the tissues make them difficult to isolate and characterize. Because of this lipopigments are generally quantified by the spectrofluorimetric method (Hammer and Braun, 1988). Isolation of lipofuscin for the first time was in 1901, by peptic digestion of human intestinal mucosa (Rosenfeld, 1901). Later procedures employed acid hydrolysis and enzymatic degradation (Moore and Wang, 1947), and sonification (Hendly et al., 1963). Less drastic separation procedures were subsequently developed which involved tissue homogenization and density gradient centrifugation that apparently produce little or no change in the lipofuscin or ceroid pigments (Siakotos et al., 1970; 1981; Palmer et al., 1986; Ng Ying Kin et al., 1983).

The purity of isolated lipopigments is usually assessed by phase-contrast and fluorescent microscopy; "pure" being the absence of debris and non-fluorescent particles; electron microscopy is also frequently employed to evaluate purity. These procedures yield about 100-200 mg of

lipofuscin per normal 40-50 year old human brain (1.2-1.4 kg) while the ceroid from the brain of a patient with Batten's disease may constitute around 5 percent of the total brain weight (Siakotos et al., 1970). Ceroid separated from the liver, kidney, pancreas and brain of 12 to 24 month old sheep with ceroid lipofuscinosis was about 1.4 mg dry mass of ceroid per gram of wet weight of tissue (Palmer et al., 1986). The fraction of lipopigment present in situ that is extracted by these procedures is apparently unknown.

The fluorophore(s) in lipofuscin is probably less than 1 percent by weight of the lipopigment mass (Wolfe, 1988).

Electron microscopy of isolated heart and liver lipofuscin shows a homogeneous population of characteristic lipopigments, unique for each organ system, while brain lipofuscin is heterogeneous. Ceroid fractions from pathologic human and canine brain show significant morphological differences according to the type of neuronal ceroid-lipofuscin (Siakotos and Munkres, 1981; Palmer, et al., 1986).

The properties of isolated lipopigments have been extensively discussed (Elleder, 1981). They have generally been reported to contain 40-70 percent by weight of proteinaceous material (Bjorkerud et al., 1964; Palmer et al., 1986) the remainder being lipid with small amounts of several metals - the levels of copper and iron are higher in ceroid than in lipofuscin (Siakotos and Koppang, 1973). Lipids that have been isolated include the lysosomal marker bis(monoacylglycero)phosphate, free fatty acids, cholesterol, ubiquinone, dolichol, and dolichol esters. Reflecting the absence of standardized isolation procedures, and the need for them to be reevaluated, was the finding in one study that over 80 percent of the lipofuscin was polymalonaldehyde (Siakotos and Munkres, 1982).

POSSIBLE MECHANISM FOR LIPOFUSCIN FORMATION

Free radical reactions are involved in lipopigment formation (Wolman, 1980, 1981). This was indicated first in 1928 when a granular, acid-fast material resembling age pigment was observed (Pinkertan, 1928) in the pulmonary parenchyma of rabbits after the intratracheal injection of cod liver oil; this oil contains high concentrations of readily oxidized polyunsaturated lipids. A similar pigment was noted in the myometrium of vitamin E deficient rats in 1936; the investigators named the pigment "ceroid" (Martin and Moore, 1936) - lipofuscin was named in 1912 (Hueck, 1912). Further studies on the effect of dietary unsaturated lipids (Danse and Verschuren, 1978; Reddy et al., 1973; Miyagishi et al., 1967), of vitamin E deficiency (Reddy et al., 1973; Miyagishi et al., 1967), and of the intraperitoneal injection of mice with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine on the formation of autofluorescent lipopigments, as well as the formation of fluorophores during the in vitro oxidation of lipids (Gutteridge, 1984; Kikugawa, 1988; Porta et al., 1988) contributed to the current acceptance of the role of peroxidation in the genesis of lipopigments. Also contributing to this acceptance are cell culture studies (Thaw et al., 1984; Ball et al., 1988) and experiments which demonstrate that vitamin E decreases the rate of lipopigment formation (Epstein et al., 1972; Tappel et al., 1973; Blackett and Hall, 1981).

In spite of numerous studies the structure(s) of the fluorophore(s) in the lipopigments is unknown (Eldred and Katz, 1988; Kikugawa, 1988; Elleder, 1981); the suggestion (Tappel et al., 1973; Feeney-Burns et al., 1980) that the fluorophore(s) are conjugated Schiff bases, $R_1N=CH-CH=CH-NH-R_2$, resulting from the condensation of malonaldehyde produced during

lipid peroxidation with amino groups of proteins and amino acids is unlikely (Eldred and Katz., 1988; Palmer et al., 1986; Kikugawa, 1988) in part because their fluorescence emission is in the range of 420-470nm whereas that of lipopigments is about 450-650nm (Porta et al., 1988). More recently it has been suggested the autofluorescence of the pigment is a property of the protein/lipid complex, rather than a chemical species significant to the pathogenesis of the pigment (Dalefield et al., 1988), while evidence has been reported to support the possibility that retinoyl complexes may be the source of the autofluorescence in Batten's disease (Wolfe et al., 1977) and lipofuscin (Eldred and Katz, 1988).

Proteolysis may also be implicated in lipopigment formation as administration of proteinase inhibitors such as leupeptin enhance ceroid formation in experimental animals (Ivy and Gurd, 1988; Ivy et al., 1984). Leupeptin is an oligopeptide endopeptidase inhibitor produced by the actinomycetes; it has a low toxicity and inhibits the most important thiol proteinases, namely cathepsins B, H, and L. The terminal aminoaldehyde group of leupeptin inhibits thiol and serine proteinases by binding semireversibly with their thiol groups (Marzella and Glaumann, 1987; Baugh and Schnebli, 1980).

Intracellular proteins are continuously synthesized and degraded (Schoenheimer, 1946; Glaumann and Ballard, 1987); turnover rates differ and are modulated by the nutritional state (Goldberg and Dice., 1974; Pine, 1972). The rate of intracellular degradation may be high. In liver, for example, the rate may vary from 0.3 to 4.5 per cent per hour (Marzella and Glaumann, 1987; the lower-limit range, 0.3-1.5 per cent, is operationally defined as basal degradation. Possible reasons for this phenomena include (Marzella and Glaumann, 1987): 1) elimination of abnormal and denatured proteins and proteins resulting from synthetic errors, 2) modulation of important metabolic pathways by removal of enzymes not needed by the cell or synthesized in excess of actual demand, for example, phenobarbital-induced cytochrome P-450 dependent enzymes are degraded in lysosomes following cessation of phenobarbital treatment, 3) post-translational processing to activate enzymes or hormones, 4) modulation of cell growth in cooperation with synthesis, 5) provision of amino acids for synthesis of proteins and for gluconeogenesis when the exogenous supply is limited, 6) involution of organs like the uterus and the mammary glands, and 7) phenotypic alterations of cells, for example, pancreatic acinus cells can lose their secretory machinery by lysosomal degradation and may undergo transformation into hepatocytes.

There are at least two major proteolytic systems in eukaryotic cells, the lysosomal system and the soluble ATP-dependent pathway (Goldberg, 1987). The latter is responsible for the rapid elimination of cytosolic proteins with abnormal structures, such as those that might result from mutation, biosynthetic errors or postsynthetic damage (Hershko and Ciechanover, 1982) while the lysosomes are apparently responsible for the most part for the degradation of long-lived proteins, fragments of membranes, and intact organelles such as mitochondria. There are at least two different types of ATP-dependent systems, the ubiquitin system in the cytosol and the vanadate-sensitive system in the mitochondria (Goldberg, 1987). The ATP-dependent proteases are most active at 7.8 pH; they have molecular weight of around 500,000. In contrast the proteases of the lysosomes are most active at acid pH's; they are glycoproteins with molecular weights of 20,000 to 40,000. It has been estimated that 30-70 percent of endogenous cell protein breakdown occurs outside the lysosomes (Ballard, 1977).

Components to be degraded are "marked" in at least one of eight ways (Stadtman, 1986) for selective removal by lysosomal and/or non-lysosomal

pathways. "Marking" increases the hydrophobicity or otherwise alters (Bohley et al., 1985; Cervera and Levine, 1986; Roseman and Levine, 1987) a component so that it is "recognized" by a protease and degraded. For example, exposure of glutamine synthetase to an oxidizing system causes inactivation, a loss of one histidine residue, and the introduction of carbonyl groups. This form of the enzyme is more hydrophilic than the native form and it is not a substrate for proteolysis. On continued exposure to the oxidizing system, a second histidine residue is lost and there is a further increase in carbonyl content. This form is significantly more hydrophobic than the native form and is very susceptible to proteolytic attack (Cervera and Levine, 1986; Roseman and Levine, 1987). The selective capacity of lysosomes for "marked" proteins, membranes and organelles may be increased by dolichols. These long chain hydrophobic polyprenols containing an unsaturated isoprene unit have a high affinity for membrane structures. They are synthesized in the microsomes and then transported for the most part to the lysosomes (Wong et al., 1982a,b). The only known function of dolichol, in the form of dolichol phosphate, is its participation as an active "carrier" of glycosyl groups in the synthesis of N-linked glycoproteins (Struck and Lennarz, 1980). The phosphorylated forms of dolichol amount to only a small fraction of total cellular dolichol. The remaining dolichol and dolichol esters may possibly serve to increase the hydrophobicity of the lysosomal surface so as to enhance recognition and uptake of cellular components marked for degradation.

In addition to "normal marking", such as by ubiquitin conjugation (Hershko, 1985), by the oxidation of amino acid residues by mixed-function oxidation systems, or by the phosphorylation of serine or threonine residues (Stadtman, 1986; 1988), cellular components may be "marked" in a more-or-less random manner by reacting with free radicals arising from non-enzymatic sources or "leaking" from enzymatic free radical reactions. Such free radical reactions may make a significant contribution to the rate of "marking" of cellular components as they go on continuously throughout the cells and tissues. The level of these reactions increases with the metabolic rate and with age (Harman, 1986; Noy et al., 1985). These free radical reactions may be partly responsible for the observed increases with age of altered proteins in the cells (Gershon and Gershon, 1970; Rothstein, 1982) - inactivated, but not readily proteolyzed, as well as for decreases in neutral protease activity with age (Stadtman, 1988); the latter in turn could contribute to the increases in altered proteins with age.

Oxidatively altered proteins are largely proteolyzed by cytosolic proteases (Stadtman, 1988). Single mitochondrial proteins may be degraded in the mitochondria (Desautels and Goldberg, 1982) while the bulk turnover of the entire mitochondria takes place in lysosomes (Glaumann et al., 1981; Pfeifer, 1978). Similarly, oxidized membrane lipids are "recognized" and removed, at least in part, by the action of phospholipase A₂ (Sevanian and Muakkassah-Kelly, 1983).

Cellular damage from free radical reactions has been present from the beginning of life (Harman, 1986). The evolution of more complex and longer living cells and organisms apparently occurred through the gradual selection and development of 1) defenses against deleterious chemical reactions, particularly against the constantly present free radical reactions - during the early part of evolution these reactions were largely initiated by ionizing radiation from the sun, later, after formation of the ozone shield and the shift to aerobic metabolism, they arose for the most part endogenously, and 2) means to repair or replace cellular components that were rendered defective by free radical reactions, or others such as hydrolysis. These processes ensured

preservation of beneficial cellular activities while still permitting changes conducive to further evolution.

Defenses that have evolved to minimize the rate of production of free radical damage include antioxidants, such as the tocopherols and carotenes, glutathione peroxidase, and superoxide dismutases, as well as cellular components more resistant to free radical attack (Joenje et al., 1985).

Measures which evolved to remove and degrade cellular components damaged by free radical reactions, or others, include the DNA repair systems, the lysosomal system, and the proteinases and peptidases of the cytosol and mitochondria. As cells become more complex the proteolytic system apparently expanded to serve other purposes as indicated above while enzymes such as phospholipase A₂ appeared to aid in the removal of oxidatively damaged lipids. Compounds resulting from these measures were then available for reuse by the cell.

Normally this recycling system works extremely well. However, there could be some slow slippage with advancing age. The rate of free radical reaction damage increases, possibly exponentially judging from the exponential increase in the chance of death with advancing age, while protease activity decreases - due, at least in part to increasing free radical damage. As a result a gradually increasing fraction of lysosomal "food" may be converted to non-digestible forms (Davies, 1988), lipofuscin, before it can be broken down to reusable components. The pigment serves as a reminder of the extreme efficiency of the cellular recycling system.

Ceroid apparently is formed when the disposal system is "overloaded" or impaired. Overloaded, for example, by increased intake of polyunsaturated fatty acids (these probably increase the rate of free radical damage to cellular components), or by a genetic defect that results in an increased rate of "marking" of one or more components of the cells. The system may be impaired, e.g., by protease inhibitors such as leupeptin or genetic defects involving lysome formation, protease activity, etc. The reason for the accumulation in ceroid-lipofuscinosis is not clear. It may be due to an increased susceptibility to lipid peroxidation. Abnormalities in the fatty acid composition of phospholipids have been reported in these diseases. Thus, in a case of the infantile syndrome (Svennerholm, 1976) the content of 22:4ω6 and of 22:6ω3 in the phospholipids of the gray matter of the brain was much lower than in the controls while that of 18:1ω9 and 20:4ω6 were proportionally much higher. Another early onset case had a 30 percent decrease in the 22:6ω3 content of phosphatidylserine, this was replaced by 18:1ω9 (Jervis and Pullarkat, 1976). A study of infantile, late infantile and adult ceroid-lipofuscinosis (Pullarkat et al., 1982) brain gray matter found that the phosphatidylserine fraction had a 35-90 percent reduction in docosahexaenoic acid (22:6ω3). Studies such as these, the recent report of increased levels of 4-hydroxynonenal (Siakotos et al., 1988), an oxidation product of 22:4ω6, in a canine model of ceroid-lipofuscinosis and the increased iron content reported in this disorder (Johansson et al., 1984) all suggest that there is some genetic defect(s) in this disorder(s) which results in increased lipid peroxidation damage and/or abnormality in phosphatidylserine which predisposes it to oxidative damage. Considering the complexity of the cellular disposal system there could be many genetic defects that result in ceroid formation in addition to those involved in the above disorders.

REFERENCES

- Armstrong, D., Koppang, N., and Rider, J. A., editors: ceroid-lipofuscinosis (Batten's disease), New York, Elsevier Biomedical Press, 1982.
- Ball, R. Y., Carpenter, K. L. H., and Hutchinson, M. J.: Ceroid accumulation by murine peritoneal macrophages exposed to artificial lipoproteins: ultrastructural observations. *Brit. J. Exper. Path.*, 69:43-56, 1988.
- Ballard, F. J.: Intracellular protein degradation, in *Essays in Biochemistry*, Vol. 13, edited by Campbell, P. N., and Aldridge, W. N., New York, Academic Press, 1977, pp. 1-37.
- Barden, H. and Brizzee, K. R.: The histochemistry of lipofuscin and neuromelanin, in *Advances in the Biosciences*, Vol. 64, *Advances in Age Pigments Research*, Totaro, E. A., Glees, P., and Pisanti, F. A., editors, New York, Pergamon Press, 1987, pp. 339-392.
- Barden, H.: The biology and chemistry of neuromelanin, in *Age Pigments*, edited by Sohal, R. S., New York, Elsevier/North Holland Biomedical Press, 1981, pp. 155-175.
- Baugh, R. J., and Schnebli, H. P.: Role and potential therapeutic value of proteinase inhibitors in tissue destruction, in *Monograph Series of the European Organization for Research on Treatment of Cancer*, Vol. 6, edited by Strauli, P., Barrett, A. J., and Baici, A., New York, Raven Press, 1980, pp. 59-67.
- Berkovic, S. F., Carpenter, S., Andermann, F., Andermann, E., and Wolfe, L. S.: Kufs disease: a critical reappraisal. *Brain*, 111: 27-62, 1988.
- Bjorkerud, S.: Isolated lipofuscin granules - a survey of a new field, in *Advances in Gerontological Research*, Vol. 1, edited by Strehler, B. L., New York, Academic Press, 1964, pp. 257-288.
- Blackett, A. D., and Hall, D. A.: Tissue vitamin E levels and lipofuscin accumulation with age in the mouse. *J. Gerontol.*, 36:529-533, 1981.
- Bohley, P., Hieke, C., Kirschke, H., and Schaper, S.: Protein degradation in rat liver cells, in *Progress in Clinical and Biological Research*, Vol. 180. *Intracellular Protein Catabolism*, edited by Khairallak, E. A., Bond, J. S., and Bird, J. W. C., New York, Alan R. Liss, Inc., 1985, pp. 447-455.
- Brizzee, K.R., and Ordy, J.M.: Cellular features, regional accumulation, and prospects of modification of age pigments in mammals, in *Age Pigments*, edited by Sohal, R.S., New York, Elsevier/North Holland Biomedical Press, 1981, pp. 101-154.
- Cervera, J., and Levine, R. L.: Mixed function oxidation of glutamine synthetase modulates the hydrophobicity of the protein and controls its susceptibility to proteolyses. *Fed. Proc.*, 45:1597, 1986.
- Dalefield, R. R., Jolly, R. D., Craig, A. S., Martinus, R. D., and Palmer, D. N.: Age pigment in the thyroid of aged horses, in *Lipofuscin-1987: State of the Art*, edited by Zs.-Nagy, I., New York, Elsevier Science Publishers, 1988, pp. 213-226.
- Danse, L. H. W. C., Verschuren, P. M.: Fish oil-induced yellow fat disease in rats. I. Histological changes. *Vet. Pathol.*, 15:114-124, 1978.
- Davies, K. J. A.: Protein oxidation, protein cross-linking, and proteolysis in the formation of lipofuscin: rationale and methods for the measurement of protein degradation, in *Lipofuscin-1987: State of the Art*, edited by Zs.-Nagy, I., New York, Elsevier Science Publishers, 1988, pp. 109-132.
- Desautels, M., and Goldberg, A. L.: Demonstration of an ATP-dependent, vanadate-sensitive endoprotease in the matrix of rat liver mitochondria. *J. Biol. Chem.*, 257:11673-11679, 1982.
- Eldred, G. E. and Katz, M. L.: Fluorophores of the human retinal pigment epithelium: Separation and spectral characterization. *Exper. Eye Res.*, 47:71-86, 1988.

- Elleeder, M.: Chemical characterization of age pigments, in Age Pigments, edited by Sohal, R. S., New York, Elsevier/North Holland Biomedical Press, 1981. pp. 203-241.
- Epstein, J., and Gershon, D.: Studies on ageing in nematodes. IV. The effect of antioxidants on cellular damage and life span. *Mech. Age. Dev.*, 1:257-264, 1972.
- Feeney-Burns, L., Berman, E. R., and Rothman, H.: Lipofuscin of human retinal pigment epithelium. *Amer. J. Ophthalmol.*, 90:783-791, 1980.
- Friede, R. L.: The relation of the formation of lipofuscin to the distribution of oxidative enzymes in the human brain. *Acta Neuropathologica*, 2:113-125, 1962.
- Friede, R. L., and Fleming, L. M.: A mapping of oxidative enzymes in the human brain. *J. Neurochem.*, 9:179-198, 1962.
- Gershon, H., and Gershon, D.: Detection of inactive enzyme molecules in ageing organisms. *Nature*, 227:1214-1217, 1970.
- Glaumann, H., Ericsson, J. L. E., and Marzella, L.: Mechanisms of intraliposomal degradation with special reference to autophagocytosis and heterophagocytosis of cell organelles. *Intl. Rev. Cytol.*, 73:149-182, 1981.
- Glaumann, H., and Ballard, F. J., editors. *Lysomes: Their Role in Protein Breakdown*. New York, Academic Press, 1987.
- Goldberg, A. L.: The ATP-dependent pathway for protein degradation in mitochondria, in *Lysosomes: Their Role in Protein Breakdown*, edited by Glaumann, H., and Ballard, F. J., New York, Academic Press, 1987, pp. 715-722.
- Goldberg, A. L. and Dice, T. F.: Intracellular protein turnover in mammalian and bacterial cells. *Ann. Rev. Biochem.*, 48:835-869, 1974.
- Gutteridge, J. M. C.: Age pigments: role of iron and copper salts in the formation of fluorescent lipid complexes. *Mech. Ageing Dev.*, 25:205-214, 1984.
- Hadjiconstantinou, M., Tijoe, S., Alho, H., Miller, C., and Neff, N. H.: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) accelerates the accumulation of lipofuscin in mouse adrenal gland. *Neuroscience Letters*, 83:1-6, 1987.
- Hammer, C., and Braun, E.: Mini-review: Quantification of age pigments (lipofuscin). *Comp. Bichem. Physiol.*, 90B:7-17, 1988.
- Hamperl, H.: Die fluoreszenzmikroskopie menschlicher gewebe. *Virchows Arch. Pathol. Anat. Physiol.*, 292:1-51, 1934.
- Hannover, A.: Mikroskopische undersogelser of nervensystemf. Kgl. Danske Videns Kabernes Selskabs. C. Naturv. og Math. Afh, Copenhagen, 10:1-112, 1842.
- Harman, D.: Free radical theory of aging: role of free radicals in the origination and evolution of life, aging, and disease processes, in *Free Radicals, Aging, and Degenerative Diseases*, edited by Johnson, Jr., J. E., Walford, R., Harman, D., and Miquel, J., New York, Alan R. Liss, 1986, pp. 3-49.
- Hendley, D.D., Mildvan, A. S., Reporter, M. C., and Strehler, B. L.: The properties of isolated human cardiac age pigment. I. Preparation and physical properties. *J. Gerontol.*, 18:144-150, 1963.
- Hermansky, F. and Pudlak, P.: Albinism associated with hemorrhagic diathesis and unusual pigmented reticular cells in the bone marrow: Report of two cases with histochemical studies. *Blood*, 14:162-169, 1959.
- Hershko, A., and Ciechanover, A.: Mechanisms of intracellular protein breakdown. *Ann. Rev. Biochem.*, 51:335-364, 1982.
- Hershko, A.: The ATP-ubiquitin proteolytic pathway, in *Progress in Clinical and Biological Research*, Vol. 180, *Intracellular Protein Catabolism*, edited by Khairallah, E. A., Bond, J. S., and Bird, J. W. C., New York, Alan R. Liss, Inc., 1985, pp. 17-31.
- Hueck, W.: Pigmenstudien. *Beitrag. Path. Anat.*, 54:68-232, 1912.
- Ivy, G. O., Schottler, F., Wenzel, J., Baudry, M., and Lynch, G.:

- Inhibitors of lysosomal enzymes: accumulation of lipofuscin-like dense bodies in the brain Science, 226:985-987, 1984.
- Ivy, G. O., and Gurd, J. W.: A proteinase inhibitor model of lipofuscin formation, in Lipofuscin-1987: State of the Art, edited by Zs.-Nagy, I., New York, Elsevier Science Publishers, 1988, pp. 83-106.
- Jervis, G. A., and Pullarkat, R. K.: Pigment variant of lipofuscinosis. Neurology, 28:500-503, 1978.
- Joenje, H., Gille, J. J. P., Oostra, A. B., van der Valk, P.: Some characteristics of hyperoxia-adapted HeLa cells. Lab. Invest., 52:420-428, 1985.
- Johansson, E., Lindh, U., Alanen, T., Westermark, T., Heiskala, H., Santavuori, P., and Elovaara, I.: Elemental profiles of blood cells in certain neurological disorders, Medical Biol., 62:139-142, 1984.
- Kikugawa, K.: Involvement of lipid oxidation products in the formation of lipofuscin, in Lipofuscin-1987: State of the Art, edited by Zs.-Nagy, I., New York, Excerpta Medica, 1988, pp. 51-68.
- Koneff, H.: Beitrag zur Kenntniss der nervenzellen in den peripheren Ganglien. Mitth. d. Naturf. Gesellsch. Bern, 44:13-14, 1886.
- Koppang, N.: Canine ceroid-lipofuscinosis - a model for human neuronal ceroid-lipofuscinosis and aging. Mech. Ageing Dev., 2:421-445, 1973/1974.
- Levine, R. L., Oliver, C. N., Fulks, R. M., and Stadtman, E. R.: Turnover of bacterial glutamine synthetase: Oxidative inactivation precedes proteolysis. Proc. Natl. Acad. Sci. USA, 78:2120-2124, 1981.
- Levine, R. L.: Oxidative modification of glutamine synthetase. I. Inactivation is due to loss of one histidine residue. J. Biol. Chem., 258:11823-11827, 1983.
- Luys, J.: Recherches sur Le Systeme Nerveux Cerebro-Spinal, Sa Structure, Ses Fonctions Et Ses Maladies. J.-B. Bailliere Et Fils, Paris, 1865.
- Martin, A. J. P. and Moore, T.: Changes in the uterus and kidneys in rats kept on a vitamin E free diet. Chem. Ind., 55:236, 1936.
- Marzella, L., and Glaumann, H.: Autophagy, microautophagy and crinophagy as mechanisms for protein degradation, in Lysomes: Their Role in Protein Breakdown, edited by Glaumann, H., and Ballard, F. J., New York, Academic Press, 1987, pp. 319-366.
- Miquel, J., Oro, J., Bensch, K. G., and Johnson, Jr., J. E.: Lipofuscin: fine structural and biochemical studies, in Free Radicals in Biology, Vol. 3, edited by Pryor, W. A., New York, Academic Press, 1977, pp. 133-182.
- Miyagishi, T., Takahata, N., and Iizuka, R.: Electron microscopic studies on the lipo-pigments in the cerebral cortex nerve cells of senile and vitamin E deficient rats. Acta Neuropathologica, 9:7-17, 1967.
- Moore, T., and Wang, Y. L.: Fluorescent pigment and vitamin E. Brit. J. Nutrit., 1:53-64, 1947.
- Munkres, K. D.: Neurospora age pigments resemble malonaldehyde polymers, in Advances in the Biosciences, Vol. 64. Advances in Age Pigments Research, edited by Totaro, E. A., Glees, P., and Pisanti, F. A., New York, Pergamon Press, 1987, pp. 165-184.
- Nagy, I. Zs.-, editor: Lipofuscin-1987: State of the Art, New York, Excerpta Medica, 1988.
- Ng Ying Kin, N. M. K., Palo, J., Haltia, M., and Wolfe, L. S.: High levels of brain dolichols in neuronal ceroid-lipofuscinosis and senescence. J. Neurochem., 40:1465-1473, 1983.
- Nichols, W. W., and Murphy, D. G., editors. DNA Repair Process. Miami, Miami Symposium Specialties, 1977.
- Noy, N., Schwartz, H., and Gafni, A.: Age-related changes in the redox status of rat muscle cells and their role in enzyme-aging. Mech. Ageing Devel., 29:63-69, 1985.
- Oliver, C.: Lipofuscin and ceroid accumulation in experimental animals, in Age Pigments, edited by Sohal, R. S., New York, Elsevier/North

- Holland Biomedical Press, 1981, pp. 335-350.
- Oliver, C. N., Levine, R. L., and Stadtman, E. R.: A role of mixed-function oxidation reactions in the accumulation of altered enzyme forms during aging. *J. Amer. Geriatrics Soc.*, 35:947-956, 1987.
- Palmer, D. N., Barns, G., Husbands, D. R., and Jolly, R. D.: Ceroid lipofuscinosis in sheep. II. The major component of the lipopigment in liver, kidney, pancreas, and brain is low molecular weight proteins. *J. Biol. Chem.*, 261:1773-1777, 1986.
- Pfeifer, W.: Inhibition by insulin of the formation of autophagic vacuoles in rat liver. *J. Cell Biol.*, 78:152-167, 1978.
- Pine, M. F.: Turnover of intracellular proteins. *Ann. Rev. Microbiol.*, 26:103-126, 1972.
- Pinkerton, H.: The reaction to oil and fats in the lung. *A.M.A. Arch. Pathol.*, 5:380-401, 1928.
- Porta, E.A.: Tissue lipoperoxidation and lipofuscin accumulation as influenced by age, type of dietary fat and levels of vitamin E in rats, in *Advances in the Biosciences*, Vol. 64, *Advances in Age Pigments Research*, edited by Totaro, E. H., Glees, P., and Pisanti, F. A., New York, Pergamon Press, 1987, pp. 37-74.
- Porta, E. A., Mower, H. F., Moroye, M., Lee Ch., and Palumbo, N. E. Differential features between lipofuscin (age pigment) and various experimentally produced "ceroid" pigments, in *Lipofuscin-1987: State of the Art*, edited by Zs.-Nagy, I., New York, Excerpta Medica, 1988, pp. 341-372.
- Porta, E. A., and Hartroft, W. S.: Lipid pigments in relation to aging and dietary factors (lipofuscins), in *Pigments in Pathology*, edited by Wolman, M., New York, Academic Press, 1969, pp. 191-235.
- Pullarkat, R., Reha, H., Patel, V. K., and Goebel, H. H.: Docosahexaenoic acid levels in brains of various forms of ceroid-lipofuscinosis, in *Ceroid-lipofuscinosis (Batten's disease)*, edited by Armstrong, D., Koppang, N., and Rider, J. A., New York, Elsevier Biomedical Press, 1982, pp. 335-342.
- Reddy, K., Fletcher, B., Tappel, A., Tappel, A. L.: Measurement and spectral characteristics of fluorescent pigments in the tissues of rats as a function of polyunsaturated fats and vitamin E. *J. Nutr.*, 103:908-915, 1973.
- Roseman, J. E., and Levine, R. L.: Purification of a protease from *Escherichio coli* with specificity for oxidized glutamine synthetase. *J. Biol. Chem.*, 262:2101-2110, 1987.
- Rosenfeld, M.: Weber das pigment der hamochromatose des darmes. *Arch. Exper. Pathol. Pharmak.*, 45:46-50, 1901.
- Rothstein, M.: Enzymes and altered protein. *Biochemical Approaches to Aging*, New York, Academic Press, 1982, pp. 213-255.
- Schoenheimer, R.: *The Dynamic State of Body Constituents*. Boston, Harvard University Press, 1946.
- Sevanian, A., Muakkassah-Kelly, S. F., and Montestrusque, S.: The influence of phospholipase A₂ and glutathione on the elimination of membrane lipid peroxides. *Arch. Biochem. Biophys.*, 223:441-452, 1983.
- Siakotos, A. N., Bray, B., Dratz, E., van Kuyk, F.; Sevanian, A., and Koppang, N.: 4-Hydroxynonenal: a specific indicator for canine neuronal-retinal ceroidosis. *Amer. J. Med. Genetics Supplement*, 5:171-181, 1988.
- Siakotos, A. N., and Munkres, K. D.: Recent developments in the isolation and properties of autofluorescent lipopigments, in *Ceroid-lipofuscinosis (Batten's disease)*, edited by Armstrong, D., Koppang, N., and Rider, J. A., New York, Elsevier Biomedical Press, 1982, pp. 167-178.
- Siakotos, A. N., and Munkres, K. D.: Purification and properties of age pigments, in *Age Pigments*, edited by Sohal, R. S., New York,

- Elsevier/North-Holland Biomedical Press, 1981, pp. 181-202.
- Siaikotos, A. N., Armstrong, D., Koppang, N., and Mulles, J.: Biochemical significance of age pigment in neurons, in *The Aging Brain and Senile Dementia*, edited by Nandy, K., and Sherwin, I., New York, Plenum Press, 1977, pp. 99-118.
- Siaikotos, A. N., and Koppang, N.: Procedures for the isolation of lipopigments from brain, heart, and liver, and their properties: a review. *Mech. Ageing Dev.*, 2:177-200, 1973.
- Siaikotos, A. N., Watanabe, I., Saito, A., and Fleischer, S.: Procedures for the isolation of two distinct lipopigments from human brain: lipofuscin and ceroid. *Biochemical Med.*, 4:361-375, 1970.
- Sohal, R. S., editor: *Age Pigments*, New York, Elsevier/North Holland Biomedical Press, 1981.
- Sohal, R. S.: Metabolic rate, aging, and lipofuscin accumulation, in *Age Pigments*, edited by Sohal, R. S., New York, Elsevier/North-Holland Biomedical Press, 1981, pp. 303-316.
- Stadtman, E. R.: Oxidation of proteins by mixed-function oxidation systems: implication in protein turnover, ageing and neutrophil function. *Trends Biochem. Sci.*, 11:11-12, 1986.
- Stadtman, E. R.: Biochemical markers of aging. *Exper. Gerontol.*, 23:327-347, 1988.
- Strehler, B. L., Mark, D. D., Mildvan, A. S., and Gee, M. V.: Rate and magnitude of age pigment accumulation in the human myocardium. *J. Gerontol.*, 14:430-439, 1959.
- Struck, D. K., and Lennarz, W. G.: in *The Biochemistry of Glycoproteins and Proteoglycans*, edited by Lennarz, W. J., New York, Plenum Press, 1980, pp. 35-83.
- Stubel, H.: Die fluoreszenz tierischer gewebe in ultraviolettem licht. *Pfluegers Arch. Gesamte Physiol. Menschen Tiere*, 142:1-14, 1911.
- Svennerholm, L.: Polyunsaturated fatty acid lipidosis: a new nosological entity, in *Current Trends in Sphingolipidoses and Allied Disorders*, edited by Volk, B. W. and Schneck, L., New York, Plenum Press, 1976, pp. 389-402.
- Tappel, A. L., Fletcher, B., and Deamer, D.: Effect of antioxidants and nutrients on lipid peroxidation fluorescent products and aging parameters in the mouse. *J. Gerontol.*, 28:415-424, 1973.
- Thaw, H. H., Collins, V. P., and Brunk, U. T.: Influence of oxygen tension, pro-oxidants and antioxidants on the formation of lipid peroxidative products (lipofuscin) in individual cultivated human glial cells. *Mech. Ageing Dev.*, 24:211-223, 1984.
- Totaro, E. A., Glees, P., and Pisanti, F. A.: Advances in the Biosciences, Vol. 64. *Advances in Age Pigments Research*, New York, Pergamon Press, 1987.
- Witkop, C. J., White J. G., Townsend D., Sedano, H. O., Cal, S. X., Babcock, M., Krumwiede, M., Keenan, K., Love, J. E., and Wolfe, L. S.: Ceroid storage disease in Hermansky-Pudlak syndrome: Induction in animal models, in *Lipofuscin-1987: State of the Art*, edited by Zs.-Nagy, I., New York, Excerpta Medica, 1988, pp. 413-435.
- Wolfe, L. S.: Comment, in *Lipofuscin-1987: State of the Art*, edited by Zs.-Nagy, I., New York, Excerpta Medica, 1988, p. 181.
- Wolfe, L. S., Ng Ying Kin, N. M. K., Baker, R. R., Carpenter, S., and Andermann, F. A.: Identification of retinoyl complexes as the autofluorescent component of the neuronal storage material in Batten's disease. *Science*, 195:1360-1362, 1977.
- Wolman, M.: Factors affecting lipid pigment formation, in *Age Pigments*, edited by R. S. Sohal, New York, Elsevier/North-Holland Biomedical Press., 1981, pp. 265-281.
- Wolman, M.: Lipid pigments (chromolipids): their origin, nature, and significance, in *Pathobiology Annual*, Vol. 10, edited by Ioachim, H.

- L., New York, Raven Press, 1980, pp. 253-267.
- Wong, T. K., Decker, G. L., and Lennard, W. J.: Localization of dolichols in the lysosomal fraction of rat liver. *J. Biol. Chem.* 257:6614-6618, 1982a.
- Wong, T. K., and Lennard, W. J.: The site of synthesis and intracellular deposition of dolichol in rat liver. *J. Biol. Chem.*, 257:6619-6624, 1982b.
- Zuckerman, B. M.: The nematode Caenorhabditis elegans as a model for rapid evaluation of cellular aging, in *Advances in the Biosciences*, Vol. 64, *Advances in Age Pigment Research*, edited by Totaro, E. A., Glees, P., and Pisanti, F. A., New York, Pergamon Press, 1987, pp.155-163.

DISCUSSION

KATZ: One of the points you emphasized was the probable central role of oxidation in the formation of age-pigment and ceroid pigments. I concur that oxidation probably does play some role because in our *in vivo* experiments with animals deficient in antioxidant nutrients we have observed acceleration of autofluorescent pigment build-up. However, we have done some *in vitro* experiments oxidizing the same tissues where we have seen lipopigment accumulation *in vivo*, and we have seen different fluorophores than those you see in age-pigment. I think, therefore, that the oxidation story might be more complex than simple chemical oxidation that you can reproduce in the test tube.

HARMAN: I think that this depends in part on what is in the cell at the time the process is taking place. In fact, when I was reviewing the literature I began to wonder how much of what is attributed to peroxidation is actually real or an artifact. For example, it is commonly believed that peroxides are present in the atherosclerotic plaques, but these findings, as well as yours, have been done after death. However, if you extrapolate the contents of peroxides from the time of analysis back to the moment of death, you would find almost a linear regression to zero. In other words, the steady-state concentration of peroxides in atherosclerotic plaques may be essentially zero. I don't know for sure how much or what percentage of what people are seen and measuring in these autofluorescent pigments is actually taking place in the cells *in vivo*, and what is taking place as a function of the extraction procedures. What I would agree is that you may get a lot of different fluorophores depending upon what has been oxidized or undergoing changes at a given time in a cell. You have to keep in mind that in analyzing a tissue you usually work under relatively high concentration of oxygen as compared with the real conditions *in vivo*, so this will make a big difference in the relative contributions of the components to the final formation of fluorophores.

KATZ: I agree with what you are saying, but I think that in order to prove a mechanism we eventually need to reproduce it in the test tube, and up to now we have not been able to do it. If we are going to demonstrate that these yellow fluorescent pigments are oxidation products, we eventually have to be able to synthesize them, and prove that oxidative reactions are responsible for their formation.

HARMAN: I agree with your last comments.