



Review Article

BIOCHEMICAL BASIS OF LIPOFUSCIN, CEROID,
AND AGE PIGMENT-LIKE FLUOROPHORES

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Abstract—Serious studies of the formation mechanisms of age-related pigments and their possible cellular influence have been hampered for a long time by discrepancies and controversies over the definition, fluorescence emission, origin, and composition of these pigments. This review discusses several critical controversies in this field and lay special emphasis on the cellular and biochemical reactions related to the formation mechanisms of lipofuscin, ceroid, advanced glycation end-products (AGEs), and age pigment-like fluorophores (APFs). Various amino compounds and their reaction with secondary aldehydic products of oxygen free radical-induced oxidation, particularly lipid peroxidation, are important sources of the fluorophores of ceroid/lipofuscin, which progressively accumulate as a result of phagocytosis and autophagocytosis of modified biomaterials within secondary lysosomes of postmitotic and other cells. Lipofuscin is the classical age pigment of postmitotic cells, while ceroid accumulates due to pathologic and experimental processes. There are good reasons to consider both ceroid and lipofuscin as materials of the same principal origin. The age-related intracellular fluorophores of retinal pigment epithelium (RPE) seems to represent a special class of lipofuscin, which partly contains derivatives of retinoids and carotenoids. Saccharide-originated fluorophores, principally AGEs formed during glycation/Maillard reactions, may be mainly responsible for the extracellular fluorescence of long-lived proteins, such as collagen, elastin, and lens crystalline. Although lipofuscin, ceroid, AGEs, and APFs can be produced from different types of biological materials due to different side reactions of essential biology, the crosslinking of carbonyl-amino compounds is recognized as a common process during their formation.

Keywords—Age pigments, Carbonyls, Ceroid, Fluorophores, Free radicals, Glycation, Lipofuscin

INTRODUCTION

The age-related, progressive accumulation of yellow-brown pigments (age pigment or lipofuscin), mainly

within postmitotic cells, is a consistently recognized phenomenon in man and animals.^{1–4} These brown pigments, which primarily emit yellowish fluorescence when excited with UV or blue light, are regarded as a hallmark of aging. Their accumulation rate in humans, as reported early by Strehler, is about 0.6% of the volume of myocardial cells per decade. The pigment may occupy up to 40% of the cytoplasmic volume in postmitotic cells of old animals, whereas large motor-neurons of human centenarians may be occupied up to 75% by these deposits.⁷

Although morphologic, pathologic, and biochemical studies of these enigmatic substances have been carried out for more than a century, the biochemistry and formation mechanisms are still a matter of debate and controversy.^{8–15}

The age-related, intracellular fluorescent pigments are classically known as “lipofuscin.” These yellow-

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brown pigmented granules have also been called yellow-pigment, lipopigment, wear and tear pigment, chromolipid, lipochrome, etc. The pigment granules were described even before the articulation of the cell theory by Rudolph Virchow (1858) and were first reported by Hannover in 1842.¹⁶ The term "lipofuscin" was initially suggested by Borst and was primarily used by Hueck (1912) to distinguish this pigment from tyrosine-associated melanin pigments.¹⁷ The term "lipofuscin" is used here, only to refer to age-related, intracellular, fluorescent pigment granules in situ.

Although lipofuscin mainly accumulates in postmitotic cells, such as neurons, cardiac muscle, and retinal pigment epithelium, a similar pigment, named ceroid, is found in cells with mitotic capacity, such as hepatocytes, smooth muscle cells, and a variety of cells of the kidney, thymus, pancreas, testis, prostate, uterus, and adrenal gland of humans and animals.^{4-5,18-19} Various examples of ceroid pigments have been reported, such as those formed in the cirrhotic livers of choline deficient rats;²⁰ in the retinal pigment epithelium of vitamin E-deficient rats;^{21,22} and in atheromatous lesions of the human aorta.^{23,24} Ceroid with a fluorescence maximum at 360/430 nm was also detected in human serum showing that the pigment may also occur extracellularly,²⁵ where it may be generated either via the direct oxidative modification of extracellular lipids, amino compounds, and carbohydrates, or through cell rupture and expulsion. During the first day after major vascular surgery, trauma, or shock, patients have been found to have increased ceroid fluorophores in their serum. The fluorophore concentration later decreases to normal levels, if there are no further complications.²⁵ Such fluorescent materials, produced as a result of various experimental and pathologic conditions and not necessarily related to aging, are defined as ceroid pigments.²⁶

Both lipofuscin and ceroid tend to accumulate in the lysosomal compartment as a result of the autophagocytosis or heterophagocytosis of modified cellular materials. We believe that the basic formation mechanisms of both lipofuscin and ceroid are the same. The difference is that ceroid forms rapidly during any period in the life in any tissue,²⁷ whereas lipofuscin accumulates only slowly within postmitotic cells and is more resistant to digestion.

Age-related fluorescence of advanced glycation end-products (AGEs) is often found extracellularly, in association with long-lived proteins, such as aged lens crystalline (cataract)^{28,29} and crosslinked collagen in such collagen-rich tissues as skin (senile elastosis), arteries, lungs, and kidneys.^{30,31}

In this review, the term "age pigments" is used in a broad sense to represent both intracellular and extra-

cellular, age-related, fluorescent material, including AGEs.

In addition to intracellular and extracellular fluorescent pigments, induced by aging or various pathologies, different fluorophores with similar fluorescent characteristics can be produced in different in vitro model systems using biological materials as substrates, for example, by lipid peroxidation, protein oxidation, and Maillard reactions. Because such fluorophores are still not well characterized and not generally accepted as parts of age pigments, they are referred to, for the moment, as "age pigment-like fluorophores" (APFs).

The relationships and definitions of lipofuscin, ceroid pigments, age pigments, AGEs, and APFs are shown in Fig. 1. Lipofuscin and AGEs are included under age pigments but not ceroid. Although fluorophores are important constituents of age pigments, not all age-associated pigmented biomolecules are fluorescent.

PHYSICO-CHEMICAL PROPERTIES OF LIPOFUSCIN, CEROID, AND APFS

The methodological background of age pigment studies

Lipofuscin, ceroid, and age pigment-like fluorophores have been studied for more than a century, from different perspectives, at different levels, and in different model systems. The most important discoveries, however, have been made in the past few decades using modern techniques. Three methods often used to characterize and quantify such pigments are: (1) transmission electron microscopy of fixed cells and tissues; (2) microfluorometry of fixed or native cells and tissues; and (3) spectrofluorometry of suspensions, isolates, and extracts of such pigments.

The technical differences between microfluorometry and spectrofluorometry are often overlooked, although the differences are important. As shown in Table 1, lipofuscin observed by microfluorometry usually is in the form of condensed, tiny granules, whereas extracted and much diluted fluorophores are measured by spectrofluorometry.

In addition, the physical characteristics of the light best used to study fluorescent age pigments are controversial. Therefore, a comparison between absorption, fluorescence emission, and visible colors is provided in Table 2. Undoubtedly, the colors of monochromatic light (similar to laser light) should be considered as the right reference for relating wavelength with color. As shown in Table 2, the visible colors of cutoff filters are different, particularly in the blue region. The colors of cutoff filters as well as most substances around us,

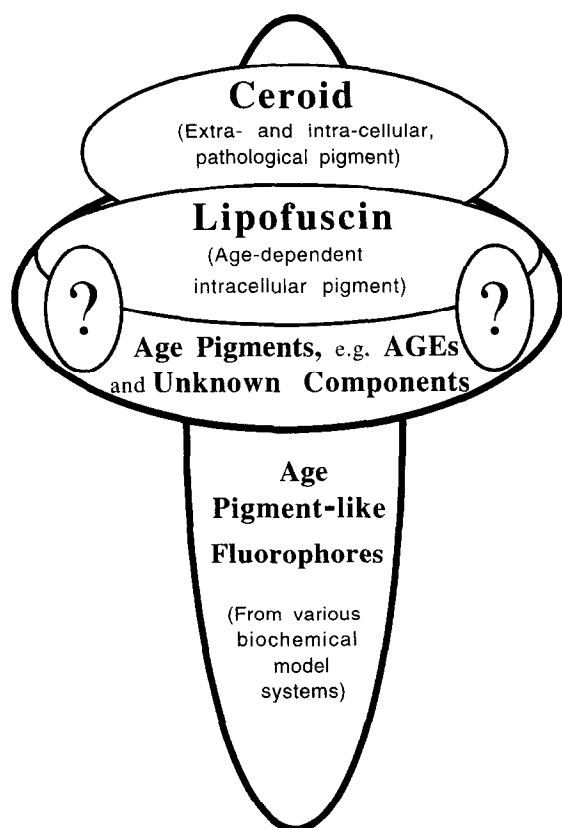


Fig. 1. Relationship among age pigment-associated fluorophores.

which are not light sources and always absorb light, show a color that has no direct bearing on emission wavelengths. As shown in Table 2, the fluorescent emission in the region between 400 to 500 nm is considered to be blue, whereas fluorescent emission > 500 nm is considered to be yellow-green to orange-red.

Morphological and microfluorometrical studies

Early studies of lipofuscin granules were mainly based on morphological and histochemical techniques. In the bright-field light microscope, lipofuscin is observed within postmitotic cells, such as neurons and cardiac myocytes, as yellowish-brown, irregularly shaped pigment granules with a distribution and size corresponding to secondary lysosomes.^{1-3,18-19} Lipofuscin granules are usually 1–5 μ m in diameter. By electron microscopy, the granules are found being bound

by a single limiting, 100 nm thick membrane and containing various vacuolar, lamellar, and osmiophilic granular substructures.

Lipofuscin is histochemically characterized by the following properties: basophilia, due to the presence of acidic groups; periodic acid-Schiff positive, probably due to the presence of carbonyl lipids or carbon chains with adjacent aldehyde groups; stainable by lipid stains such as Sudan black B; and stainable by reagents specific for proteins.^{3-5,18,33}

However, the reported biochemical composition of lipofuscin varies significantly between different tissues and species, and also between different studies. According to Björkerud, three major components are recognized: lipids (20 to 50%), proteins (30 to 50%), and hydrolysis-resistant residues (10 to 30%).³⁴

The presence of various saccharides as components of lipofuscin granules has recently been reported on the basis of histochemical studies. Lipofuscin granules, isolated from human brains, were reported to contain mannose, glucose, galactose and their amine complexes.³⁵⁻³⁸ Glycoconjugates, detected by lectin histochemistry, were also reported to be important constituents of lipofuscin and ceroid.^{39,40} Such sugars, however, may exist in the form of glycoproteins or glycolipids in the lysosomal aggregates and may not contribute to the autofluorescence of lipofuscin.

One of the most pronounced characteristics of lipofuscin is its yellowish-orange autofluorescence when excited with UV or blue light by microfluorometry. However, the fluorescence emission of lipofuscin is very complicated. Hyden and Lindström reported dual emission peaks of 440–460 and 530–560 nm in the earliest spectral study using microfluorometry.⁴¹ Based on the yellow-orange emission characteristics observed in the microfluorometer, it was later suggested by Strehler that the “fluorescence emission extends from about 500 . . . into . . . 630” nm.¹ Further studies with microfluorometric technique have often supported that lipofuscin in situ emits light-yellow, greenish-yellow, or yellow-orange fluorescence (500–640 nm) when excited with UV or blue light.^{15,42-50}

It is worth noting that formaldehyde-fixation of cells and tissues is often used in the studies of lipofuscin autofluorescence in situ.^{51,52} The crosslinking or conjugation of formaldehyde with various cellular bioamines, forming Schiff bases, is the very same mech-

Table 1. Differences in the Techniques of Microfluorometry and Spectrofluorometry

	Materials under Study	Accuracy of Spectral Data	Light Path Length	Special Advantages
Microfluorometry	solid (concentrated)	distorted, or limited, by filters used	microns	fluorescence visible
Spectrofluorometry	solutions (very diluted)	accurate	mm–cm	quantitative and sensitive

Table 2. Comparisons of Absorption and Fluorescent Emission with Visible Color

Absorption up to . . . (nm) (Using Cutoff Filters as Reference)	400	450	500	550	> 580
Color	No Color	Greenish Yellow	Yellow	Orange Yellow	Red to Purple
Fluorescence emission (nm) (Using Monochromator as Reference)	<400	450	520–580	600	> 630
Color	Violet	Violet to Deep-Blue	Greenish Yellow	Orange Yellow	Red to Purple

anism that also underlies lipofuscin formation. Thus, the fixation process per se may produce new ceroid/lipofuscin-like fluorescence.⁵³ The fluorescent feature varies, depending on the different bio-amines that happen to be present in the tissues tested. Therefore, the interpretation of fluorescent spectra from formaldehyde-treated “ceroid-lipofuscin” is risky.

The combination of different excitation and emission filters for microfluorometry is critical when observing and characterizing the fluorescence spectrum of lipofuscin. Katz and colleagues reported that when sections from RPE samples were excited using a 340–380 nm bandpass filter in combination with a 430 nm barrier filter, only a fraction of the RPE lipofuscin granules were faintly visible in the fluorescence microscope, showing a whitish-yellow color.²¹ The fluorescence, however, was enhanced by a 355–425 nm/460 nm filter combination. When the same sample was studied using a 390–490 nm/515 nm filter combination, the intensity of the lipofuscin fluorescence was greatly enhanced, exhibiting a golden-yellow fluorescence. The last filter combination was, therefore, considered the most effective setup. Similar finding was also reported by Dowson and Harris.⁵⁴ They noticed that a 390–490 nm/515 nm filter combination produced the greatest intensity of yellowish lipofuscin autofluorescence, in comparison with various other combinations of excitation and barrier filters.

Although excitation and emission bandwidths affect the characterization of fluorescence spectra, specific discussions on this matter are rare in the literature.

Isolation, extraction, and spectrofluorometric studies of lipofuscin, ceroid, and APFs

To characterize the physico-chemical properties of age pigments, lipofuscin granules have been isolated and extracted using different techniques. Extensive studies on the separation of lipofuscin were performed by Björkerud and Siakotos.^{34,55–56} The isolation of lipofuscin is complicated due to significant differences in the density of lipofuscin particles of different origin.⁵⁶ Even more difficulties were encountered following attempts to dissolve lipofuscin pigments.⁹ Resistance to extraction by different solvents was often

found to be enhanced during increased “maturation” of the lipofuscin.⁵⁷ A variety of extraction techniques using water, chloroform/methanol, ethanol, ethanol/diethylester, sodium dodecylsulfate (SDS), etc., have been documented in the literature.^{22,59–63} Chloroform/methanol (2:1) is so far the most popular solvent for lipofuscin extraction.^{58–59,63–64} The solubility and extractability of lipofuscin and APFs in water and organic solvents were comprehensively reviewed by Porta, Tsuchida, and others at the end of the 1980s.^{26,65} Because there have been no significant improvements during the past few years, this subject will not be further discussed here. However, the use of SDS or lithium dodecylsulfate, which has the advantage to dissolve proteinous compounds, has added new possibilities.^{61,66}

Although lipofuscin in situ generally shows yellowish autofluorescence when studied by microfluorometry, the fluorescence maxima of lipofuscin-extracts studied by spectrofluorometry are consistently reported to be in the blue (400–500 nm) region.⁶⁷ Such blue emission is also often obtained from fluorophores produced in various model systems, as long as the lipofuscin derived from retinoids and carotenoids is not included. From spectrofluorometric studies, some examples of accurate fluorescent spectra of lipofuscin-extracts and APFs, showing blue emission between 400 and 500 nm, are listed in Table 3.

Concerning the typical yellow-orange fluorescence of lipofuscin in situ, the consistent observation of blue fluorescence in extracts of the same tissue remains a striking discrepancy. It is noteworthy (as also listed in Table 1), that intact and condensed lipofuscin is studied by microfluorometry, whereas lipofuscin-extracts and APFs are diluted many hundred times when studied by spectrofluorometry.

Eldred and colleagues suggested that the blue fluorescence of extracted lipofuscin may result from instrumental bias due to different photon sensitivities of the photomultipliers used.⁹³ To obtain true spectra, they suggested careful corrections of both the excitation and the emission spectra. It should be noted, however, that their studies were mainly carried out using human RPE cells and are, thus, perhaps not of general validity. After successful correction of the fluorescence spectrum

Table 3. Fluorescence Maxima of Lipofuscin Extracts and APFs by Spectrofluorometry

Author	Year [References]	Sources	Excitation/Emission (nm)
Chio et al.	1969 [68]	Biochemical	365/470
Siakotos et al.	1970 [69]	H.s.* (brain)	360/450
Dillard & Tappel	1971 [70]	Biochemical	360/430
Bidlack & Tappel	1973 [71]	Biochemical	370/445
Fletcher et al.	1973 [58]	Biochemical	360–380/440–470
Siakotos & Koppang	1973 [57]	H.s. (brain et al.)	360/450
Tappel et al.	1973 [72]	Lipid peroxidation	350–370/445–465
Malshet et al.	1974 [73]	Various	355–380/430–480
Miquel et al.	1974 [74]	<i>D. melanogaster</i>	370–375/440–450
Desai et al.	1975 [59]	Rat	370/440–450
Siakotos & Armstrong	1975 [75]	H.s. in general	350–360/435–450
Trombly et al.	1975 [76]	H.s. (testes)	345–350/485–490
Csallany & Ayaz	1976 [60]	Rat	365/435
Shimasaki et al.	1977 [77]	Rat	360/470
Donato & Sohal	1978 [78]	<i>M. domestica</i>	365/435
Miquel et al.	1978 [79]	Mice (testis)	360–380/440–470
Munkres & Rana	1978 [80]	<i>P. anserina</i>	365/425
Koster & Slee	1980 [81]	Rat (liver)	350/430
Sohal & Buchan	1981 [82]	<i>M. domestica</i>	380/455
Shimasaki et al.	1982 [83]	Lipid peroxidation	360/430
Ettershank et al.	1983 [63]	<i>E. superba</i>	350/430
Monnier & Cerami	1983 [29]	Human lens	375/440
Allen et al.	1984 [84]	<i>M. domestica</i>	365/435
Ettershank	1984 [64]	<i>E. superba</i>	350/420
Monnier et al.	1984 [85]	Human collagen	370/440
Fukuzawa et al.	1985 [86]	Biochemical	351/448
Nicol	1987 [87]	<i>M. norvegicus</i> etc.	350/438
Jones & Lunec	1987 [88]	Protein oxidation	360/454
Sell & Monnier	1989 [89]	Human collagen	360/460
Maeba et al.	1990 [61]	Biochemical	360/430
Yin & Brunk	1991 [90]	Biochemical	377/430, etc.
Yin	1992 [91]	Biochemical	350/430
Giulivi & Davies	1993 [92]	Hemoglobin oxidation	325/410

* Abbreviations used in Table 3: *D. melanogaster* = *Drosophila melanogaster*, *E.* = *Euphausia*, *H.s.* = *Homo sapiens*, *M. domestica* = *Musca domestica*, *M. norvegicus* = *Meganyctiphanes norvegicus*, *P.* = *Podospora*.

of RPE lipofuscin and that of peroxidized RPE homogenates, they found that peroxidation-related fluorophores are different from those of RPE lipofuscin. Whereas the corrected fluorescent maxima of RPE lipofuscin were in the region of yellow-orange-red (500–640 nm), the corrected maximum of peroxidation-related fluorescence (using RPE homogenate as substrate) was in the blue region, i.e., an emission maximum at 440 nm when excited at 360 nm.⁹⁴ These data indicate that diluted peroxidation products do not show yellow-red fluorescence, and also suggest that the photomultiplier correction has limited significance, particularly in the blue region. These results led them to argue that “the autofluorescent products of lipid peroxidation may not be lipofuscin-like.” Instrumental correction has not provided a solution to the discrepant fluorescence data. Instead, it challenges the current biochemical interpretation of the formation of lipofuscin, particularly the widespread idea that this pigment is formed under the influence of oxidative stress.

Recently, an alternative explanation of the discrepant fluorescent data was suggested by Yin and Brunk,

who found a significant concentration-dependent fluorescence shift.⁹⁵ A variety of proposed age pigment fluorophores, including 1,4-dihydropyridines, Schiff bases and MDA polymers, all demonstrated a potential for spectral shifts. With increasing concentrations, the emission peaks of these fluorophores shifted from blue (400–490 nm) to golden-yellow or even orange-red (500–600 nm). The study of this concentration-dependent quenching and shift of autofluorescence has been an underdeveloped domain in spectrofluorometry.^{96,97} Early views on such shifts were mainly based on investigations of metachromatic properties, leading to the opinion that they were due to a transition of the fluorophores from a monomeric to a polymeric (stacked) form at high concentration.⁹⁸ Accordingly, such metachromatic effect was considered to be (1) a combination of a chromotrope with the resonance forms of the dye, and/or (2) an orderly dye aggregation by the formation of new intermolecular bonds between adjacent dye molecules.⁹⁹ We found that this explanation is not correct for several simple fluorophore solutions. If the quenching is due to the formation of dimers or poly-

mers, the spectra of a fluorophore solution at a given concentration should be consistent, i.e., when measuring a fluorescent polymer, alterations of light path length should alter the intensity but not the fluorescence maximum. In fact, the opposite occurred. The spectra of all fluorophores tested at high concentrations shifted their fluorescence maxima to shorter wavelengths when a 1.0 cm quartz cuvette was replaced by 0.1 or 0.01 cm cuvettes.¹⁰⁰

It is important to point out that a concentration-dependent fluorescence shift is usually not observable during microfluorometry using an epi-illumination system, in which the effective light path length is of the order of a few microns. A quenching, or inner filtering effect, occurs only at very high fluorophore concentrations, e.g., when the lysosomotropic weak base acridine orange is accumulated within the acidic lysosomal compartment. When lipofuscin is accumulated to a very high concentration within secondary lysosomes during aging, the fluorescence red-shift may become substantial, though, even by epifluorometry. These findings add further support to the concept that discrepancies in age pigment fluorescence data are due to various quenching conditions during measurements using different techniques.

We conclude, therefore, that when accumulated at high concentrations and/or mixed with other light absorbing materials, none of the age-pigment fluorophores can exhibit their original, individual fluorescence maxima, typically observed at low concentrations. The general occurrence of quenching-related fluorescence shifts should be taken into consideration when comparing *in vitro* biochemical with *in situ* cytochemical data on age pigments.

The concentration-dependent fluorescence shift was initially studied with spectrofluorometers using relatively thick (1×1 cm) cuvettes.¹⁰⁰ However, to obtain ideal fluorescent spectra by spectrofluorometry, it is necessary to use a very short light path (about $10 \mu\text{m}$), which simulates the conditions of microfluorometry while avoiding its defects. The results of preliminary studies using this special technique, that was recently performed in our laboratory, indicate that such studies may open new approaches for the clarification of the discrepancies in lipofuscin fluorescence that have been encountered over the years.

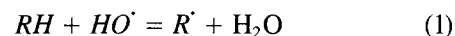
OXYGEN-DERIVED RADICALS, LIPID PEROXIDATION, AND THEIR RELATION TO LIPOFUSCIN, CEROID, AND APFs

Oxidative stress, mitochondria, and lysosomes

The free radical theory of aging, proposed by Harman in 1956, is now one of the leading theories ex-

plaining the biochemical basis of ceroid and lipofuscin formation.¹⁰¹ It was developed into its present form following the discovery of superoxide dismutase by McCord and Fridovich in 1969,¹⁰² which established the modern science of oxygen-related free radicals in biology and medicine.

When electrons are added singly to oxygen, different oxygen intermediates are created. Two of these intermediates, the superoxide anion radical, $\text{O}_2^{\cdot-}$, and particularly the hydroxyl radical, HO^{\cdot} , are highly reactive, capable to initiate oxidation of lipids, proteins, and DNA, which may lead to further cellular and biological damage:



Many biomolecules, particularly polyunsaturated fatty acids (PUFAs), are very susceptible to oxygen radicals. Because all cellular membranes are basically lipid bilayers, the integrity of which is essential to life, free radical-induced lipid peroxidation processes have become one of the major foci of research in the life sciences during the past several decades. Oxidative stress-induced lipid peroxidation was the earliest suggested origin for the formation of lipofuscin and APFs.^{68,70}

The electron transport chain of mitochondria is the main locus for the formation of reactive oxygen species, including hydrogen peroxide.^{103,104} In the presence of ferrous iron (catalytically active form), hydrogen peroxide may turn to form the very aggressive hydroxyl radicals (HO^{\cdot}), which is known as Fenton reaction.

Lysosomes are known to digest intra- and extracellular material following autophagocytosis and endophagocytosis with the help of some 40 lytic enzymes. Recently, heavy metals such as iron have been found in the lysosomes of most cells as a consequence of normal autophagocytic degradation of various metalloproteins. In macrophages and Kupffer cells, iron may be enriched during the phagocytosis of red blood cells or cell debris. A lysosomal pool of "free iron" may, thus, be created for short periods during digestion within the lysosomal compartment.^{105,106} Iron and oxygen free radical-related peroxidation may, thus, occur inside lysosomes, resulting in the formation of cross-linked biocomplexes that are not degradable by the lysosomal hydrolytic enzymes. Brunk and co-workers proposed that the accumulation of undegradable oxidized material (mainly from autophagocytosis of mitochondria) in lysosomes may be essential for lipofuscinogenesis in cells, particularly in long-lived postmitotic cells.¹⁰⁵ Ivy and co-workers discovered that the administration of inhibitors of lysosomal proteinase (e.g., E-64, leupeptin, and chloroquine) induced a dose-

and time-dependent accumulation of ceroid/lipofuscin-like dense lysosomal bodies in a variety of cells in vivo.^{107–109} A similar increase in ceroid-lipofuscin was reported when proteinase inhibitors were added to rat cardiac myocytes in culture.¹¹⁰ These findings are also compatible with the concept that biological materials, during a prolonged lysosomal degradation, may become more susceptible to peroxidation by intralysosomal, iron catalyzed, oxidative reactions.¹⁰⁵ However, in Ivy's experiments, the inhibition of lysosomal proteinases resulted in only a transient increase in the amounts of ceroid lipofuscin.¹¹¹ The incomplete lysosomal digestion, due to various causes, may underlie the accumulation of brown fluorescent end products, i.e., lipofuscin.

Lipid peroxidation and formation of lipofuscin, ceroid, and APFs

After the initiation of lipid peroxidation by reactive oxygen species or their iron complexes (reaction 1), lipid hydroperoxides and cyclic peroxides are formed (Fig. 2), which further decompose, or degrade, into various saturated or unsaturated carbonyl compounds (mainly aldehydes). Unsaturated aldehydes are often cytotoxic or genotoxic. A majority of them are precursors of fluorescent products. Among them, malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are the most extensively studied ones. In 1969, Tappel and his colleagues first reported that the lipid peroxidation product, malondialdehyde, reacts with amino acids to form age pigment-like fluorophores.⁶⁸ This finding provided an attractive link between free radical damage and aging. The PUFA peroxidation-related formation of MDA and the ensuing formation of APFs are shown in Fig. 2.

Reactions between MDA and various amino acids under strong acidic conditions yield fluorescent compounds with emission in the 450–470 nm region when excited by ultraviolet light (Fig. 2, left panel). Infrared and mass spectral analysis indicate that the fluorescent compounds are conjugated Schiff bases with an aminoiminopropene structure ($—N=CH—CH=CH—NH—$).⁸

Kikugawa et al. reported that the reaction of MDA with primary amines, at neutral pH and 37°C, produces another group of fluorescent compounds, 1,4-dihydropyridine-3,5-dicarbaldehydes (Fig. 2, right panel).¹¹² These compounds exhibit fluorescent emission maxima round 435–465 nm when excited at 365–405 nm. Although these fluorescent maxima are similar to those of aminoiminopropene, several other properties are different. Nevertheless, formation of Schiff bases is a fundamental chemical process during fluorophore formation at both acidic and neutral pH.

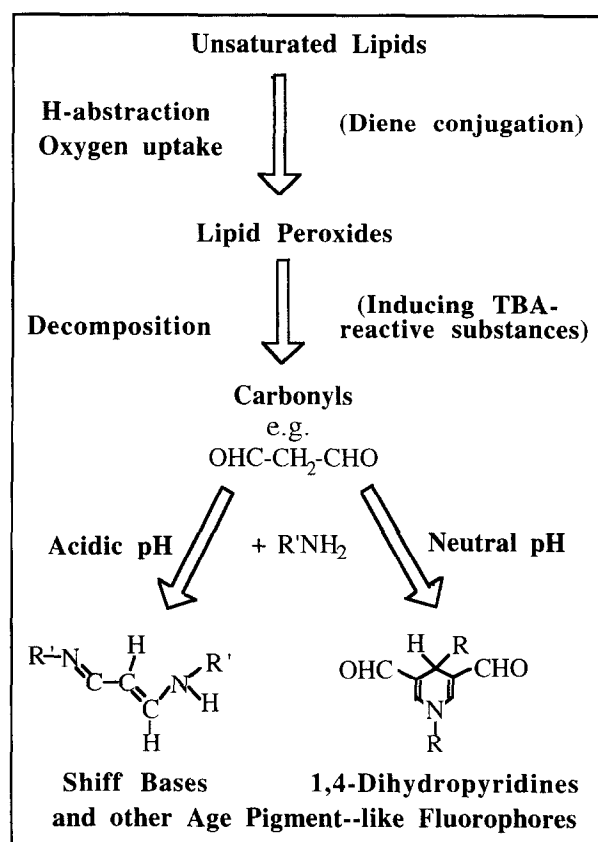


Fig. 2. Lipid peroxidation-induced MDA and APFs (emission at 425–475 nm).

In the absence of primary amines, Gutteridge et al. obtained other MDA-related fluorescent products, which he suggested to be polymerized MDA.¹¹³ Because of the presence of a large amount of amino compounds within organisms, such nonnitrogen fluorescent polymer may not be of significance in vivo.

HNE formed during Fe^{2+} -dependent lipid peroxidation was also, like MDA, found to react with microsomes, mitochondria, and amino phospholipids, producing fluorescent materials.¹¹⁴ The spectral characteristics of these fluorophores (ex./em., 360/430 nm) are identical to extracts of oxidatively damaged cellular organelles and lipofuscin-extracts.^{58,70,81}

Numerous APFs have been obtained following reactions between primary amines and secondary lipid peroxidation products other than MDA and HNE,^{48,86,115–120} e.g., the lipid peroxidation products, 2,4-hexadienal and 2,3-butanedione,¹²¹ 12-keto-oleic acid,⁷⁸ and 2,4-decadienal.¹²⁰ Monofunctional aldehydes are also reported contributing to the formation of APFs.¹¹⁷ Most short carbon-chain aldehydes, such as formaldehyde, acetaldehyde, and glutaraldehyde, can also react with biomolecules to form fluorescent products.^{87,122–124}

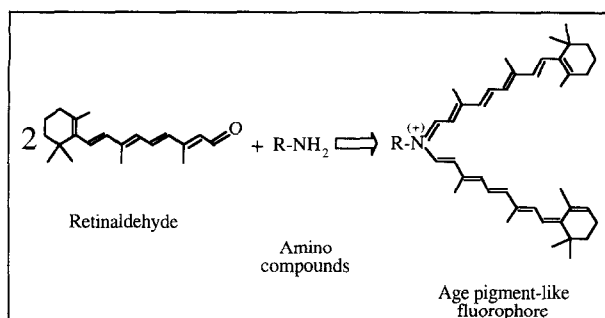


Fig. 3. An orange-emitting APF; lipofuscin of retinal pigment epithelium.

Although these APFs vary greatly in their origin, composition, and properties, such as solubility, pH effect, etc., these fluorescent compounds all appear to form through a common conjugation-related crosslinking process between amino compounds and different carbonyls, particularly unsaturated carbonyls.

POLYENE-RELATED APFs

Several polyenic compounds (polyisoprenoids), e.g., retinoids, carotenoids and dolichols, have been reported to be responsible for the formation of ceroid and lipofuscin. It should be noted that both retinoids and carotenoids have a number of conjugated double bonds in their molecular structure, that are important chromogens and, thus, responsible for the absorption and fluorescence of these compounds. Their fluorescent derivatives often exhibit fluorescence maxima in the yellow-red (500–630 nm) region, even when diluted solutions are assayed spectrofluorometrically.

Retinoids and retinal pigment epithelium

Lipofuscin within the retinal pigment epithelium have been carefully studied by Eldred, Katz, and their colleagues.^{49,125} The formation of RPE lipofuscin is substantially enhanced during vitamin E deficiency and the pigment is shown to contain derivatives of retinoids.¹²⁶ A variety of fluorescent components have been detected in retinal pigment epithelium, showing fluorescence in the yellow to red region (500–640 nm) by both microfluorometric and fluorometric techniques.⁵⁴ A major fluorophore of this pigment was recently identified by Eldred and Lasky as a reaction product between retinaldehyde and ethanolamine (Fig. 3). Again, this is a Schiff base and a product formed between an unsaturated carbonyl and an amino compound.¹⁴

More recently, Szveda reported that rat liver ceroid-lipofuscin extracted with methanol contains re-

tinyl palmitate, showing fluorescence between 450 and 600 nm.¹⁵

Carotenoids and dolichols

Carotenoids have also been found to give rise to lipofuscin-like fluorophores. The spectra of carotenoid-related fluorophores from brain and heart were studied by Tatarian and co-workers as early as in the 1970s.⁴⁴ Similarities in the fluorescence of lipofuscin granules and β -carotene in oil has been demonstrated by microfluorometry. Considering that oxidative-cleavage of one carotene molecule results in two retinaldehyde molecules, it is possible that carotenoids may be transformed to APFs via the retinaldehyde-amine conjugation.

Dolichol is a product of the mevalonate pathway, by which carotene is also synthesized. Wolfe and colleagues reported that dolichols (long chained polyisoprenols) are significantly increased in brains from patients with Alzheimer's disease compared to age-matched controls¹²⁷ and also in the cerebral cortex of patients with infantile, juvenile, and adult types of neuronal ceroid-lipofuscinosis.¹²⁸ Increased amounts of phosphorylated dolichols were also found in the heart and kidney. Therefore, the accumulation of dolichol might also be related to advanced aging. However, because the enic structure of the isoprene chain of dolichols is not conjugated, the spectral properties of dolichols (absorption at 210 nm) are not lipofuscin-like. Dolichols are neither brownish in color nor do they exhibit lipofuscin-like fluorescence. The reaction between the dolichaldehydes, which are oxidation prod-

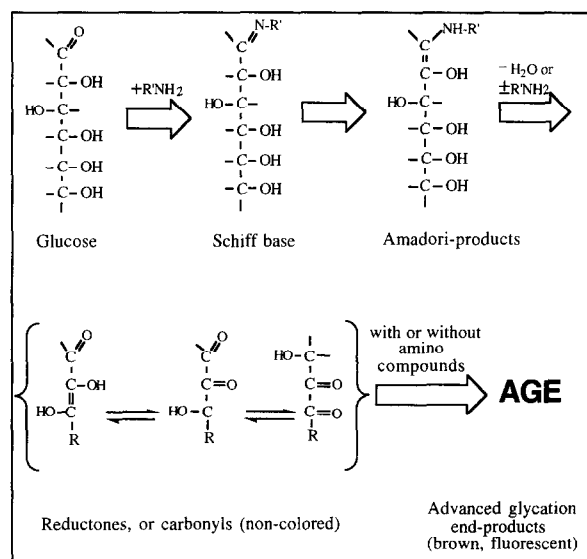


Fig. 4. Nonenzymatic glycosylation (Maillard) reactions.

ucts of dolichols, and lysine results in fluorescent products with excitation/emission maxima around 320/390 nm.²⁶ This is quite different from lipofuscin-specific fluorescence. Nevertheless, dolichols, as pointed out by Wolfe, “are distinctive constituents of ceroid and lipofuscin.”²⁶ The reasons for the accumulation of undegradable dolichols in secondary lysosomes have not yet been clarified.¹²⁹

CARBOHYDRATE-RELATED AGE PIGMENTS AND AGES

Whereas studies on oxygen radical-induced peroxidation mainly concern the lipid-associated APFs, reactions between various carbohydrates, particularly reducing sugars, and amino compounds have been found to give rise to another group of age- or time-dependent APFs.³² Such APFs are mainly formed during glycation or Maillard reactions and their formation is greatly accelerated during diabetes mellitus.^{29,31,85}

Glycation and AGEs

Nonenzymatic glycosylation, also called glycation (Maillard) reaction, represents a group of biochemical reactions between reducing sugars and amino compounds. Glycation was first studied by Louis Camillard Maillard and was initially reported in 1912.¹³⁰ When reducing sugars, such as glucose or fructose, react with amino compounds, Schiff bases are created. Schiff bases are then converted to ketoamine compounds, called Amadori products (Fig. 4). These products, although fairly stable, may undergo further degradation through deamidation or dehydration, resulting in a variety of unsaturated carbonyls, such as furfural and deoxyones (reductones), which are analogues of secondary lipid peroxidation products.^{131,132} Although the presence of oxygen and transition metals, such as iron, may greatly accelerate these processes and result in complicated oxidation products, the glycation reaction *per se* is not oxygen-dependent.¹³¹ This point is critically stressed by Yin and Brunk in a recent review.¹³³ Further reactions between such di- and multifunctional carbonyls (DMcarbonyls) and amino compounds result in the formation of the yellow-brown AGEs.^{32,87} Maillard reactions were comprehensively reviewed by Hodge as early as 1953. A schematic comparison of the fluorophore formation, showing similarities and differences between Maillard and oxidative reactions, is given in Fig. 5.

Similar to lipid peroxidation-induced APFs, AGEs are also a big family of mostly nitrogenous, cyclic and conjugated complexes, or polymers, which may be free or bound to proteins or other biomolecules as summarized in Fig. 5. Possible AGE complexes, such as car-

boxymethyllysine (CML),¹³⁴ pyrimidines and 2-(2-furoyl)-4-(2-furannyl)-1H-imidazole (FFI),¹³⁵ pyrrolines or pyrroles,¹³⁶ pentosidines,^{137,138} naphthyridinium salts also called crosslines,^{139,140} and their protein complexes have been reported. Typical AGEs are mainly extracted from human collagen and lens cataracts, which show blue autofluorescence.⁸⁵ This is analogous to the fluorescent feature of extracts of lipofuscin, ceroid and various oxidized biomaterials. However, several AGE-related compounds, such as CML, pyrroline or pyrroles, do not emit lipofuscin-like fluorescence because they do not contain largely conjugated (more than four conjugated double bonds), lipofuscin-specific fluorophores. Pentosidine, having a fairly different fluorescent excitation/emission maximum at 335/385 nm, is considered to be one of the major AGE fluorophores by some scientists,³² but not by others.¹⁴⁰ The fluorescent characteristics of FFI (370/440 nm) and crosslines (360/450 nm) agree well with typical AGE and lipofuscin-like fluorescence,^{105,139–140} although it has been argued that the formation of FFI might be an assay artifact.^{141,142}

Incubation of fructose with albumin is found to produce much more intense AGE fluorescence than glucose.^{143–145} The formation mechanism and the fluorophore composition for fructation are not yet clear.

Generally speaking, biochemical identification of AGEs is still at an early stage. The general process shown in Fig. 5 would, in our opinion, be a helpful guide for biochemists who wish to endeavour themselves in this field.

Aging phenomena related to Maillard reactions are believed to be due to carbonyl-induced crosslinking of various biomolecules, particularly proteins. The high blood glucose in diabetes is the main reason for the increase in glycated hemoglobin and, thus, ensue various diabetic late complications, e.g., atherosclerosis and nephrosis. Glycation reactions also contribute to many remarkable, age-related changes in nonrenewable tissues. During aging, the human lens crystalline becomes progressively less soluble and forms yellow chromophores and fluorophores resulting in brownish-yellow cataracts.²⁹ With age, there is a loss of elasticity in the skin, lungs, arteries, and joints, that is attributable mainly to Maillard reaction-dependent crosslinking of collagen in these collagen-rich tissues.⁸⁵

The terms glycation and Maillard reaction are often used interchangeably to represent reactions between different carbohydrates and amino compounds. Whereas glycation reactions mainly refer to reactions between reducing sugars and amino compounds, Maillard reactions have been often referred to have a broader coverage including ascorbic acid-related browning.¹³¹

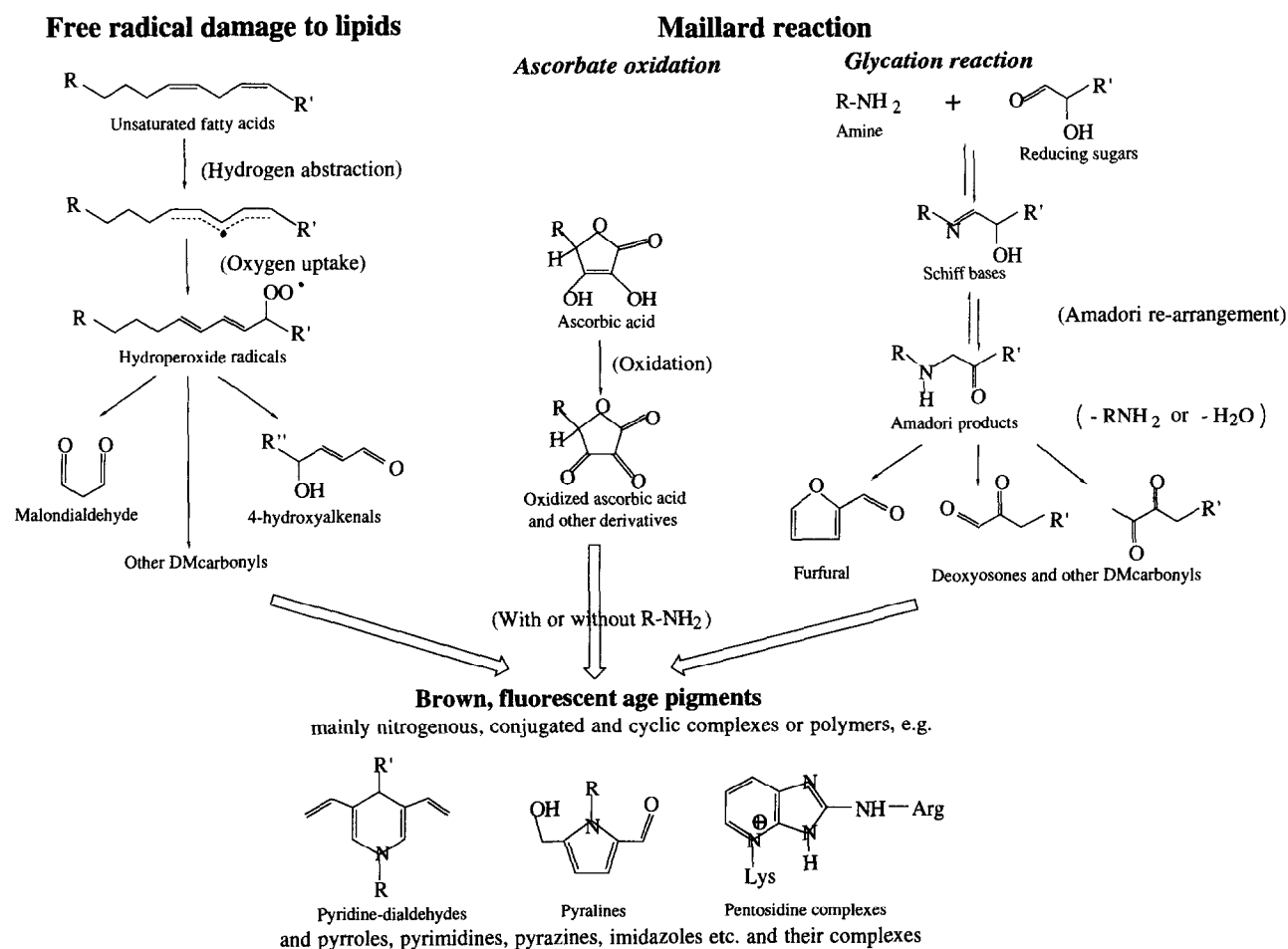


Fig. 5. Formation mechanisms of di- and multifunctional carbonyls (DMcarbonyls) and further reactions.

Ascorbic acid and APFs

In addition to glycation reactions, browning of food and beverages in relation to ascorbic acid oxidation is an area well studied by food scientists. Nevertheless, the relationship between ascorbate browning and the formation of fluorescent age pigments was understood only recently.^{90,91}

Ascorbic acid and iron compounds have been used widely to induce free radical-related lipid peroxidation and the formation of APFs.^{70-71,94,146-147,147-149} On the other hand, Ortwerth, Bensch, and colleagues reported that ascorbic acid, being more reactive than glucose, contributes to the color changes of aging lenses by inducing extensive protein crosslinking.¹⁵⁰⁻¹⁵³ The fluorescent maxima of these polymers (350/450 nm) are similar to those of homogenates of human cataract lenses.

Recently, Yin and Brunk reported that lipofuscin-like fluorophores and nonenzymatic browning were observed in aqueous solutions of oxidized ascorbic acid and reaction products between ascorbic acid (AsA) and

various amino acids.^{90,91} AsA autoxidation and its ensuing polymerization were suggested to be responsible for the formation of the particular fluorophores. These reaction products exhibit fluorescence emission (410–480 nm) similar to those of extracts from lipofuscin-rich tissues. The fluorescence intensity was found to be directly proportional to AsA concentration. Addition of H_2O_2 considerably increases the rate of the fluorescence formation, while the iron chelators, DTPA and desfer-

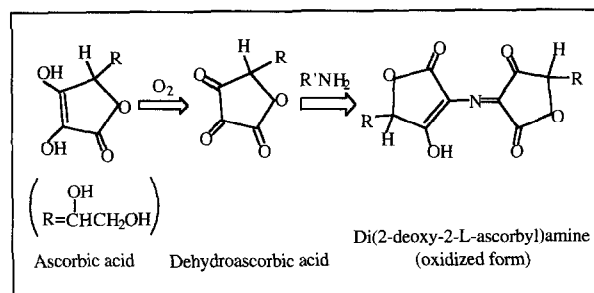


Fig. 6. APFs from ascorbic acid oxidation and amino compounds.

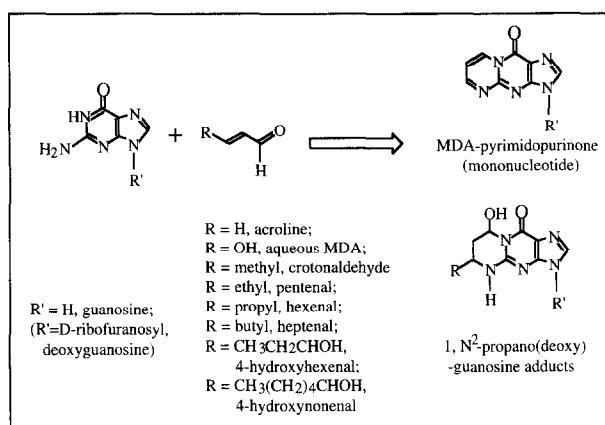


Fig. 7. Reactions involving $\alpha\beta$ -unsaturated carbonyls and nucleotides.

rioxamine, inhibit the fluorophore development by preventing iron-catalyzed AsA oxidation. These findings suggest that results from previous studies dealing with AsA-related, oxygen free radical-dependent, lipofuscin-like fluorophore formation may have been partly misinterpreted. Because oxidative derivatives of ascorbic acid may directly contribute to the blue fluorescence emission that obviously is not derived only from oxidized lipids and proteins.

APFs from ascorbate oxidation (Fig. 6), lipid peroxidation (Fig. 2), and glycation (Fig. 4) are summarized in Fig. 5.

OXIDATIVE MODIFICATION OF PROTEINS AND APFS

Proteins, peptides, and amino acids play a central role in APF formation and may all be involved in the formation of Schiff bases, when primary amines react with reducing sugars or aldehydes from lipid peroxidation, which further develop to form APFs.

In general, direct oxidation products of proteins are usually not considered to be lipofuscin but rather ceroid, probably due to differences with respect to their rate of formation. Proteins may be rapidly oxidized, giving rise to ceroid, whereas lipofuscin accumulates only slowly with age. It is still necessary to be aware of the fluorescent characteristics of these fluorophores in proteins when age pigment studies are carried out with protein-rich samples.

Protein oxidation and fluorescent products

Most proteins and protein-related biomolecules are fluorescent in the 310–350 nm UV region. The native protein fluorescence is mainly from the aromatic amino



Fig. 8. Fluorescence microscopy (ex. ≈ 450 nm; barrier filter 530 nm) of artificial lipofuscin obtained by prolonged UV-irradiation of rat liver mitochondrial fraction. (Magnification $\times 600$).

acids, tryptophane (excitation/emission, 287/348 nm) and tyrosine (275/303 nm), and slightly from phenylalanine (260/282 nm).¹⁵⁴ Oxidized aromatic amino acids, however, show fluorescence in the 400–470 nm region.

Pirie reported that when free tryptophane and bovine lens proteins were exposed to sunlight, blue fluorescent N'-formylkynurenine (330/440 nm) was produced.^{155,156} She suggested that this photo-oxidative change would be related to the formation of brown cataract. Wickens and Lunec found that when immunoglobulin or lipid-free albumin were exposed to ultraviolet irradiation or free radical sources, fluorescent products appeared with a characteristic lipofuscin-like emission (360/454 nm).^{88,157–158} They, therefore, suggested that oxidatively modified proteins, particularly the tryptophan product kynurenine, are a part of age pigments.⁸⁸

Dityrosine, having a characteristic fluorescence (315/410 nm), was reported as a specific oxidative product of tyrosine and tyrosine-containing proteins.¹⁵⁹ As reported recently, crosslinked products of oxidized calmodulin emit fluorescence with a maximum at 400 nm,¹⁶⁰ and oxidatively damaged red blood cells and hemoglobin exhibit fluorescence at 325/410 nm due to dityrosine formation.⁹² However, the tyrosine-related fluorophore is basically different from lipid peroxidation-related APFs. These two types of fluorophores were successfully separated and determined by Kikugawa and colleagues using HPLC technique.¹⁶¹ Dityrosine fluorescence was noticed as an early product in an oxidative stress model system in our laboratory. Such dityrosine fluorescence (325/410 nm) was later replaced by lipofuscin-like fluorescence around 350/430 nm (unpublished observation).

Oxidative damage of proteins, assayed as increase in protein carbonyls, has been extensively studied by Stadtman and colleagues.^{162–164} Among various amino acids, tyrosine, tryptophane, phenylalanine, histidine, and cysteine are the ones most vulnerable to oxidative modification. Protein carbonyls have been reported significantly increased during aging.¹⁶⁴ The amount of protein carbonyls varies from about 2 nmol per milligram protein in young subjects to about 4 nmol (amounts to 20–30% of the total cellular protein) in old subjects. Stadtman and co-workers suggest that "in reality, 40 to 50% of the proteins in old individuals might be present in an oxidatively damaged form." Further possible reactions between protein carbonyls and amino compounds, that may result in APFs, have not yet been carefully studied.

Ceroid lipofuscinosis and protein-rich "lipofuscin"

Ceroid-lipofuscinosis, also called Batten's disease, is a group of inherited lysosomal storage diseases of

young humans and animals that result in blindness, seizures, dementia, and premature death. This group of diseases was so named because of the excessive accumulation of fluorescent lipopigments resembling ceroid and lipofuscin.¹⁶⁵ Palmer, Jolly, and co-workers reported that ovine ceroid-lipofuscin is a protein-rich (70%) material that is dominated by subunit c of mitochondrial ATP-synthase.¹⁶⁶ Recently, this protein-rich material was found not to be fluorescent.¹⁶⁷ Although the absence of lipofuscin-like fluorescence of subunit c is not explained, it should be noted that non-fluorescent, protein-rich accumulations are manifestations of many other age-related diseases, including cerebral amyloidosis in Alzheimer's diseases, islet amyloidosis in type II diabetes mellitus, and various amyloidosis associated with chronic inflammatory diseases such as tuberculosis, rheumatoid arthritis, and chronic osteomyelitis.¹⁶⁸ The degree of oxidative alteration of lipids, carbohydrates, and especially proteins, as described previously, is important for the fluorescence of such protein-rich age-associated accumulates.

APFs AND THEIR RELATION TO NUCLEIC ACIDS

APFs were reported being created when the nucleotide bases, guanine, cytidine, and adenine were incubated with malondialdehyde.^{169,170} Similarly, fluorescent DNA products were observed when calf thymus DNA was reacted with peroxidizing arachidonic acid at 37°C for 76 h.¹⁷¹ The formation of fluorescent products was suggested due to the formation of Schiff bases, following reactions between DNA amino groups and carbonylic peroxidation products.^{171,172} Pyrimido(1,2-a)purin-10(3H)-one nucleosides were considered to be the main fluorescent products (360/460 and 358/500 nm) of reactions between MDA and nucleic acids (Fig. 7).^{173–175} Other workers, however, failed to confirm

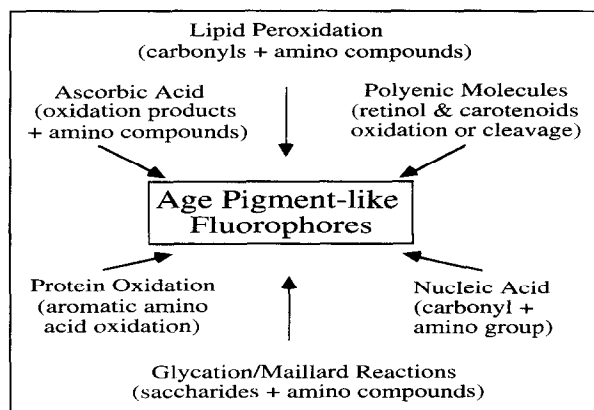


Fig. 9. Various origins of lipofuscin, ceroid, AGEs and age pigment-like fluorophores.

these studies.^{147,176} Fujimoto and colleagues reported that fluorescent products was obtained only when DNA was incubated with ferric iron and ascorbic acid.¹⁴⁷ They concluded that malondialdehyde is not the major component that reacts with amino groups of DNA to induce fluorescent APFs. It should be noted that experimentally it is difficult to discriminate the fluorescence of MDA-nucleotides adducts from that of MDA-polymers. Other recent studies on reactions between nucleotides (e.g., deoxyguanosine) and lipid peroxidation products have been presented without mentioning anything about fluorescence.¹⁷⁷⁻¹⁸⁰ Typical reactions involving $\alpha\beta$ -unsaturated carbonyls and nucleotides are summarized in Fig. 7. It is interesting to note that most $\alpha\beta$ -unsaturated aldehydes are able to react with nucleotides, typically guanosine or deoxyguanosine, forming cyclic complexes. A functional OH group, adjacent to the $\alpha\beta$ -double bond of 4-hydroxyhexenal, 4-hydroxynonenal, and aqueous MDA, are common to these reactive $\alpha\beta$ -unsaturated aldehydes.

Moreover, the addition of xanthine and guanine to cultured colon carcinoma cells results in the formation of ceroid/lipofuscin-like fluorescent materials.¹⁸¹ MDA-deoxyguanosine fluorescent adducts (340/518 nm) have been also reported in isolates from rat liver.¹⁸²

Even though the genotoxic, mutagenic, and carcinogenic effects of unsaturated aldehydes, such as HNE, MDA, and deoxyosones are well documented,¹⁸³⁻¹⁸⁶ studies on their reactions with nucleotides with the formation of APFs are rare. Rather than producing fluorophores, most reactions between nucleotides and reactive carbonyls seem to result in nonfluorescent chromogens which, nevertheless, may still represent part of ceroid-lipofuscin.

APFs MAY BE FORMED FROM MOST BIOLOGICAL MATERIALS

Recently, we have exposed a variety of cellular subfractions and other biomolecules to UV light in a sterile (laminar air-flow) hood. Ceroid/lipofuscin-like fluorophores were produced from all of them (Fig. 8). It seems that most bio-materials tend to form brown, fluorescent complexes after being subjected to oxidative stress, unless protected by antioxidative defence systems. A sequential formation of TBARS, protein carbonyls, and ceroid/lipofuscin-type autofluorescence was found during cysteine-stimulated oxidation of a rat liver lysosomal-mitochondrial fraction supporting the idea that ceroid-lipofuscin is formed due to peroxidation of cellular material under intralysosomal degradation.¹⁸⁷

Taken together, as shown in Fig. 9, APFs can be produced from a variety of biological materials including lipids, proteins, carbohydrates, carotenoids, ascorbic acid, and, possibly, nucleic acids. Oxidative stress can strongly accelerate the formation rate of APFs. The crosslinking between carbonyls and amino groups may represent a process common to the formation of ceroid/lipofuscin fluorophores from various sources.

A review of the different origins of lipofuscin, ceroid, AGE, and APFs points to the thermodynamically favoured, time-dependent, carbonyl-protein crosslinking reactions as being the most likely mechanism. Oxidative stress, glycation reactions, protein crosslinking, and other biological side reactions all seem to contribute to the formation of age pigment-related fluorophores. The carbonyl-related toxic reactions with amino groups of biomolecules seem to be a group of gradual, partially reversible, universal, and inevitable side reactions in biological systems.

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ABBREVIATIONS

AGEs—advanced glycation end-products

APFs—age pigment-like fluorophores

AsA—L-ascorbic acid

CML—carboxymethyllysine

FFI—2-(2-furoyl)-4-(2-furanyl)-1H-imidazole

HNE—4-hydroxynonenal

MDA—malondialdehyde

PUFA—polyunsaturated fatty acid

RPE—retinal pigment epithelium

SDS—sodium dodecylsulfate

TBARS—thiobarbituric acid reactive substances