A role for vitamin A in the formation of ocular lipofuscin

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Abstract

***Background*—Lipofuscin granules in the retinal pigment epithelium are lipid protein aggregates which are thought to represent the lifelong accumulation of the non-degradable end products from the phagocytosis of photoreceptor outer segments. Given the increasing evidence for a key role for vitamin A in the formation of ocular lipofuscin, the fluorophores generated by reacting vitamin A with lipid were assessed.**

***Methods*—Reaction mixtures consisting of vitamin A (retinol) or its aldehyde (retinal) and (a) isolated rod outer segments, (b) the lipid extract of rod outer segments, (c) protein, or (d) liposomes were incubated at either pH 4.5 or 7.0 for up to 42 days. The fluorescence characteristics and mobility of the chloroform soluble fluorophores generated were compared with those extracted from purified human lipofuscin. Finally, the effect of lysosomal degradation on fluorophores generated in the above mixtures was assessed.**

***Results*—Major spectral changes were observed when ROS or liposomes were incubated with retinal. These changes were pH dependent and did not occur if retinal was replaced with retinol. A number of the fluorophores generated exhibited similar fluorescence characteristics and chromatographic mobility to those of lipofuscin. Neither the presence of protein nor exposure to lysosomal enzymes had any effect on the spectral profile or fluorophore mobility of the fluorophores generated.**

***Conclusions*—These results suggest that some of the chloroform soluble fluorophores of lipofuscin are formed as a direct reaction product of retinal and lipid.**

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Post-mitotic cells of various aging tissues accumulate non-degradable lipid protein aggregates within lysosomal bodies.[1] The generic name 'lipofuscin' or 'age pigment' has been given to this lipophilic material which constitutes a heterogeneous group of substances with characteristic physicochemical properties, including a natural yellowish-green fluorescence when excited with short wavelength light.[2] Accumulation of this pigment is greatest in metabolically active post-mitotic cells and the presence of lipofuscin has been associated with age related changes in bodily function as well as a variety of pathological conditions (for example, neuronal ceroid lipofuscinosis, cardiac hypertrophy, Ménière's disease, cirrhosis of the liver and age related macular degeneration).[3] While the origin, function, and fate of lipofuscin has remained an enigma for over 100 years, there is growing evidence correlating lipofuscinogenesis with the age related peroxidation of cellular components, especially lipids.[4]

In the eye, lipofuscin is present in the retinal pigment epithelium (RPE).[5] Topographically, maximal accumulation occurs in the posterior pole and by the eighth decade lipofuscin granules occupy up to 19% of the cytoplasmic volume in human macular RPE cells by 80 years of age.[6] It is believed that lipofuscin accumulation reaches a critical level within the RPE at which point lipofuscin based damage supersedes the rate of repair mechanisms thus contributing, at least in part, to the onset of age related macular degeneration (AMD).[5 7]

Unlike other cells in the body, in which lipofuscin occurs through the autophagic breakdown of intracellular organelles,[1] the major substrate for lipofuscin formation in the RPE is the undegradable end products resulting from the phagocytosis of photoreceptor outer segments.[8–10] It is widely accepted that lipofuscinogenesis in the RPE occurs via lipid auto-oxidation and aldehyde/amine cross linking reactions.[11 12] However, there is growing evidence for a key role for vitamin A, and its oxidative breakdown products, in the formation of lipofuscin within the RPE. Firstly, the accumulation of lipofuscin in rats has been shown to be dependent on the dietary level of vitamin A; rats maintained on a vitamin A deficient diet developed only minimal autofluorescent material while rats raised on a high vitamin A diet accumulated significant amounts of lipofuscin-like fluorescence within their RPE.[13] Secondly, the development of such fluorescence in the RPE of rats exposed to (a) oxidising conditions or (b) protease inhibitors was dependent on the availability of dietary vitamin A.[14 15] Thirdly, when Eldred and Katz separated the chloroform soluble fraction of lipofuscin into 10 fluorophores by thin layer chromatography,[16] two of these fluorophores were found to comigrate with retinol and retinyl palmitate; a third, the major orange emitting fluorophore of lipofuscin, has been identified as an amphoteric quaternary amine that arises as a Schiff's base reaction product of retinaldehyde and ethanolamine.[17]

This study aimed to consolidate our current, limited knowledge of the role of vitamin A in lipofuscin formation. In particular, we have

compared the fluorescence characteristics of purified human lipofuscin with reaction mixtures consisting of vitamin A (retinol) or its aldehyde (retinal) and (a) isolated rod outer segments, (b) the lipid extract of rod outer segments, (c) protein (BSA); between 45% and 80% of RPE lipofuscin may consist of protein (Wassell and Boulton, unpublished), or (d) liposomes. Reactions were undertaken at both pH 4.5 and 7.0 in order to respectively mimic the acid environment of the lysosomal vacuome and the relatively neutral extralysosomal environment. Finally, we assessed the effect of lysosomal degradation on the above mixtures since ocular lipofuscin is considered to be formed from a reaction between the products of autophagy and ROS within the lysosomal system of the RPE.

Materials and methods

PREPARATION OF SUBSTRATES

Isolation of rod outer segments (ROS) and preparation of ROS extract

ROS were isolated as previously described[10] from light adapted bovine eyes obtained from a local abattoir. Eyes were transported on ice and all preparations were undertaken within 2 hours after slaughter. Isolated ROS were suspended in universal buffer (28.6 mM citric acid, 28.6 mM KH_2PO_4, 28.6 mM boric acid, 28.6 mM diethylbarbituric acid adjusted to the appropriate pH with 0.2 M NaOH) at pH 4.5 or 7 to give a final concentration of 2×10^7 ROS/ml.

ROS lipids were isolated using a modification of the method of Folch *et al* developed to extract lipids from animal tissues.[18] ROS at 2×10^7/ml were extracted twice with 250 µl 2:1 chloroform:methanol and 350 µl ddH_2O. The pooled chloroform phases were dried down under nitrogen and resuspended in universal buffer at pH 4.5 or 7 by sonication for use in the incubation experiments described below.

Liposome preparation

Liposomes were prepared as described by Hope *et al*.[19] In brief, phosphatidyl choline, phosphatidyl serine, and phosphatidyl ethanolamine were mixed in a round bottomed flask with dry chloroform/methanol (4/1 v/v); 1 ml of each lipid (at 20 mg/ml chloroform) was used per liposome preparation. The solvent was removed by rotary evaporation at 60°C to leave a thin film of lipid on the flask. This was transferred to a water bath at 60°C, the lipid layer rehydrated with 3 ml PBS and vortexed, in the presence of 20 µm glass beads, until all the lipid was resuspended. The multilamellar vesicles generated were extruded a total of four times through two stacked 25 mm polycarbonate filters of pore size 0.1 µm, under pressure of approximately 200 psi, resulting in liposomes ranging in diameter from 109 to 132 nm. A volume of 10 µl of the preparation was taken for sterility testing and the remainder stored at 4°C under nitrogen in the absence of light until required.

Preparation of retinal pigment epithelial (RPE) extract

Bovine eyes were obtained from a local abattoir. Eyes were transported on ice and all preparations were undertaken within 2 hours of slaughter. Following removal of the anterior segment, vitreous, and neural retina, RPE cells were detached from the eye cup using a fine camel hair brush, and transferred into universal buffer at either pH 4.5 or pH 7. The cells were sonicated on ice for 3×10 second bursts and cellular debris pelleted by centrifugation at 7700 *g* for 10 minutes. The supernatant was filtered through a 0.2 µm filter and the protein content of the filtrate determined by the BioRad protein assay. Extract volume was adjusted to contain 40 µg protein/ml by addition of universal buffer at the appropriate pH before storage at −20°C until use. The specific activities of the lysosomal enzymes β-N-acetylglucosaminidase and acid phosphatase in the RPE extract were 0.23 (SD 0.01) and 0.15 (0.01) U/mg protein respectively. Enzyme activities were assayed as previously described.[20]

Isolation of a lysosomal enzyme enriched fraction from bovine liver

Bovine liver was obtained from a local abattoir. Tissue was transported on ice and all preparations were undertaken within 2 hours of slaughter. A lysosomal enzyme enriched fraction was obtained by subcellular fractionation, based on the method of de Duve *et al*.[21] In brief, bovine liver was blended in homogenising medium (0.25 M sucrose, 5 mM TRIS-HCl, 1 mM EDTA) using a Sona blender. This mixture was subject to four centrifugation steps: (i) 700 *g*, 10 minutes, (ii) 11 300 *g*, 3 minutes, (iii) 27 200 *g*, 7 minutes, and (iv) 141 500 *g*, 60 minutes, retaining 1.5 ml samples and noting the volume of both the supernatant and pellet after each step. The lysosomal enriched fraction was identified by determining the specific activity of two lysosomal enzymes, acid phosphatase and β-N-acetylglucosaminidase, in each fraction as previously described.[20] This fraction was sonicated (3×10 second bursts) and centrifuged at 11 300 *g* for 20 minutes. The supernatant was sterilised by sequential filtration. The filtrate was aliquoted (100 µl) and stored at −20°C until required. The final specific activity for β-N-acetylglucosaminidase and acid phosphatase in the liver extract was 1.3 (0.01) and 0.46 (0.02) U/mg protein respectively.

Isolation of human lipofuscin

Lipofuscin granules were isolated and purified from human RPE (10 eyes, donor age 60–69 years) for use as standards for fluorescence spectroscopy and thin layer chromatography (TLC) as previously described.[22]

REACTION MIXTURES FOR STUDYING LIPOFUSCINOGENESIS

A list of the different reaction mixtures used is given in Table 1. The final concentration of each component in the reaction mixtures is as follows: ROS, 1×10^7/ml; ROS extract, equiva-

Table 1 List of different reaction mixtures

Substrate alone	*Substrate + retinal or retinol*
ROS	ROS
ROS extract	ROS extract
BSA	BSA
Liposomes	ROS + BSA
Retinal	ROS extract + BSA
Retinol	Liposomes
	Liposomes + BSA

All substrates were incubated for at least 7 days; incubations with liposomes were carried out for up to 42 days. ROS = rod outer segments; BSA = bovine serum albumin.

lent to 1×10^7 ROS/ml; BSA, 1.75 mM; retinol, 1.75 mM; retinal, 1.75 mM; liposomes − 6.7 mg/ml of each of phosphatidyl serine (PS), phosphatidyl choline (PC), and phosphatidyl ethanolamine (PE). ROS, ROS extract, and BSA were prepared in universal buffer; the liposomes in PBS. Retinal and retinol were prepared in methanol and diluted 1:20 in the universal buffer of the reaction mixture (the equivalent concentration of methanol was added to appropriate controls). Experiments were conducted at a final pH of 4.5 and 7.0. All incubations were carried out at 37°C in capped 1.5 ml Eppendorf tubes in the absence of light. Materials were prepared under standard laboratory lighting (fluorescent strip lights) and all experiments were repeated at least once.

In each of the incubations, 100 µl samples of freshly prepared or incubated material were taken at the varying time points, up to 7 days for most experiments but up to 42 days in the case of liposome containing samples, and stored under nitrogen at −20°C. Analysis was performed at the end of each experiment by fluorescence spectroscopy and TLC.

The effect of reactant concentration

The limited availability of reactants may have the potential to limit fluorophore formation. To test this hypothesis, experiments were repeated at a very high and a low concentration of BSA or vitamin A (that is, 17.5 and 1.75 mM). The resultant fluorophores were compared spectrophotometrically and by TLC.

Effect of lysosomal extracts on fluorophore formation from ROS

Lysosomal modification of ROS has been proposed to be a necessary prerequisite for the generation of reactive groups capable of forming lipofuscin-like fluorophores. To test this hypothesis, ROS were incubated with (1) a crude lysosomal preparation from RPE cells or (2) a lysosomal enriched preparation from the bovine liver for up to 42 days. In brief, equal volumes (200 µl) of ROS (2×10^7/ml) and lysosomal extract in universal buffer at pH 4.5 or pH 7.0 were incubated at 37°C in capped 1.5 ml Eppendorfs in the absence of light. Every other day for 8 days and then at subsequent weekly intervals, 50 µl of extract were added to the mixture. The samples were pelleted by centrifugation (1900 *g* for 10 minutes) between additions and a 50 µl aliquot removed to maintain a constant volume. Samples (100 µl), taken at days 0, 7, 21, and 42, were stored under nitrogen at −20°C before analysis. Controls were (1) ROS alone and (2) RPE extract alone incubated at 37°C, at both pH values, and topped up with the appropriate buffer. In some experiments vitamin A (1.75 mM final concentration; either retinol or retinal) in methanol was added to the reaction mixture. Methanol was included in the appropriate control tubes.

ANALYSIS OF FLUOROPHORES

Stored 100 µl aliquots of freshly prepared and incubated material were thawed; 20 µl was taken for fluorescence spectroscopy and the remaining 80 µl processed for TLC.

Fluorescence spectroscopy

Fluorescence spectra were obtained from the different preparations using a Perkin Elmer fluorescence spectrophotometer, model LS-50B, equipped with FL data manager. In brief, the 20 µl aliquot was suspended in 2 ml PBSA and the spectral profile determined using an excitation wavelength of 364 nm, monitoring emission at 570 nm; in each case, slit width was 10 nm and scan speed 240 nm/min. To minimise internal quenching effects, the concentration of each solution was adjusted such that the optical density at 514 nm was less than 0.1.[23 24] The resultant spectral profiles were corrected to allow for instrumental bias; excitation spectral correction was performed automatically by the computer software; emission spectra using the quantum counter quinine sulphate.

Extraction of autofluorescent material

The 80 µl aliquots were extracted twice by sequential addition of 250 µl 2:1 v:v chloroform:methanol (containing 10 mg/ml butylated hydroxytoluene) followed by 350 µl ddH_2O, vortexing samples for 5 seconds between additions.[16 18] Phases were separated by centrifugation at 15 000 *g* for 3 minutes and isolated using a pipette. Material from each extraction step was pooled, the chloroform phase dried down under nitrogen and resuspended in 10 µl chloroform. The methanol/water and interfacial material were freeze dried and resuspended in 20 µl PBSA for analysis by fluorescence spectroscopy.

Separation of chloroform soluble fluorophores by TLC

Aliquots (10 µl) of the chloroform phase of each sample were spotted onto a high performance silica gel TLC plate equipped with a preadsorbant spotting zone. Plates were placed in a Stimultan chamber which had been pre-equilibrated for 30 minutes with the primary solvent system described by Eldred and Katz[16] and allowed to develop for 20 minutes. Plates were dried and then photographed using an Olympus OM-1 camera fitted with a Tamron 90 mm lens and Elite-400 colour film, illuminating with a 366 nm light source. UV background was minimised using a standard Hoya UV filter together with a Kodak Wratten 2E, a Kodak CC20Y, a Lee 81EF, and a Lee 103 filter.

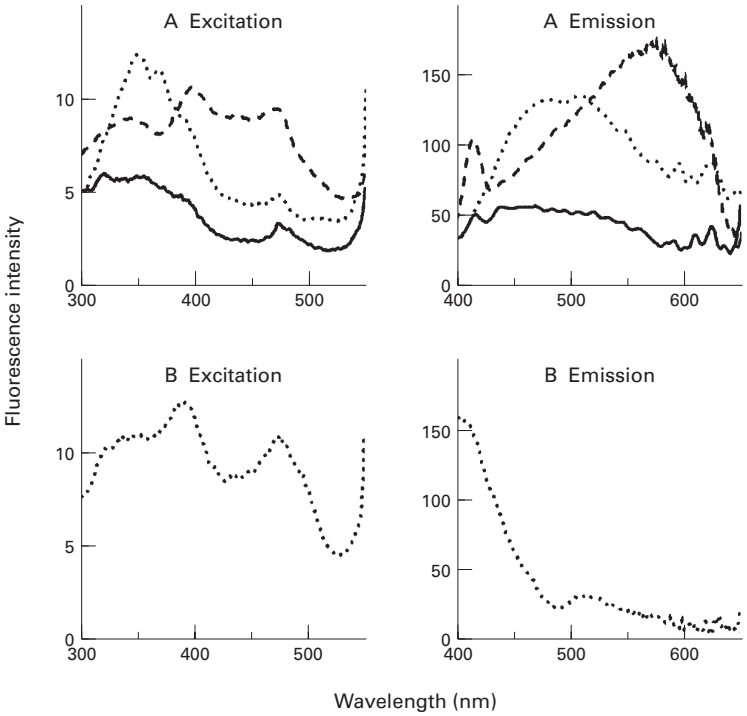

Figure 1 Spectral profile of fluorophores formed by incubating retinal with ROS. Retinal incubated with ROS at pH 7 produced long wavelength emitting fluorophores (A); no such fluorophores were formed at pH 4.5 (B). Spectra shown are of freshly prepared (—) and incubated (. . .) mixtures of retinal and ROS and are representative of a typical experiment. Similar spectral profiles were obtained in the presence of BSA. A typical spectral profile of lipofuscin isolated from five 60 to 69 year old donors (– –) is shown for comparison.

Results

Major spectral changes were observed when ROS or liposomes were incubated with retinal. These changes were pH dependent and did not occur if retinal was replaced with retinol. A number of the fluorophores generated exhibited similar fluorescence characteristics and chromatographic mobility to those of lipofuscin.

INCUBATING ROS, BSA, LIPOSOMES, RETINAL, OR RETINOL ALONE

ROS (both intact and extracted) initially formed a creamy suspension which eventually changed colour in a pH dependent manner after 7 days' incubation to bright red or light pink at pH 4.5 or 7 respectively. No change in spectral profiles or TLC banding pattern occurred during the incubation period, though both excitation and emission spectral peak heights and intensity of the TLC bands had increased following incubation.

Solutions of BSA or liposomes gradually yellowed during the incubation period in a manner which was independent of reaction pH. This yellowing was associated with increases in intensity of both the excitation and emission spectral peaks but no changes were observed in the position of these peaks and TLC profiles before and after incubation.

Addition of retinol or retinal to universal buffer initially resulted in the formation of a creamy white suspension. The visible reaction of the two forms of vitamin A then differed markedly. With retinal, the suspension darkened to form a deep purple precipitate; for retinol, this precipitate was orange/brown. These changes may reflect the formation of auto-oxidised and/or polymerised retinoid derivatives.[25 26] This colour change was pH dependent; occurring within minutes at pH 4.5 and by 2 hours at pH 7; changes were reflected by a number of peak shifts in both the excitation and emission spectra. Upon extraction, both coloured products were completely chloroform soluble and by TLC analysis retinal and retinol produced a purple or dark orange/brown smear, respectively.

REACTION OF ROS WITH RETINOL AND RETINAL

In general, incubation of ROS with retinal resulted in a pH dependent generation of long wavelength, weakly emitting, yellow/orange fluorophores with lipofuscin-like characteristics. These fluorophores were only observed at pH 7 and their formation was not affected by the inclusion of BSA in the reaction mixture. No long wavelength emitting fluorophores or yellow/orange bands were seen when retinol was used in place of retinal. Incubation with retinol produced only minor changes in spectral profiles and TLC showed that only yellow or blue emitting fluorophores were present.

The products of incubating retinal with ROS showed some similarities in fluorescence properties to that of lipofuscin (Figs 1 and 2). These similarities were most evident for samples incubated at pH 7 (Fig 1A). The fluorophores formed were excited at 355, 392, and 477 nm. These corresponded to the fluorophores of lipofuscin which excited at 351, 394, and 474 nm (Fig 1A). Emission spectra were more difficult to compare although some long wavelength emitting fluorophores, a characteristic of lipofuscin-like fluorophores, could be observed. TLC analysis showed four weakly emitting yellow/orange fluorophores (R_f s = 0.38, 0.45, 0.6, 0.74) which had similar chromatographic mobility to four of the seven fluorophores of lipofuscin (R_f s = 0.36, 0.43, 0.64, 0.71) (Fig 2, Table 2). Some of these fluorophores were also present in freshly prepared solutions, although at reduced intensities (Fig 2).

For samples incubated at pH 4.5, although no long wavelength emitting fluorophores were detected (Fig 1B), TLC revealed the presence of two yellow fluorophores with R_f s = 0.38 and 0.6 (Fig 2, Table 2).

The inclusion of BSA in the ROS-retinal reaction mixture did not affect the spectral profile or TLC banding (Table 2). No long wavelength emitting fluorophores or yellow/orange bands were detected when retinal or retinol were incubated with BSA in the absence of ROS.

Replacement of intact ROS with ROS extract in the incubation mixtures of retinal and BSA also resulted in the generation of long wavelength emitting fluorophores and six yellow/orange fluorescent bands. Two of these bands (R_f = 0.34 and 0.42) had similar appearance and chromatographic mobility to two of the fluorophores of lipofuscin. When compared with spectral profiles of fluorophores

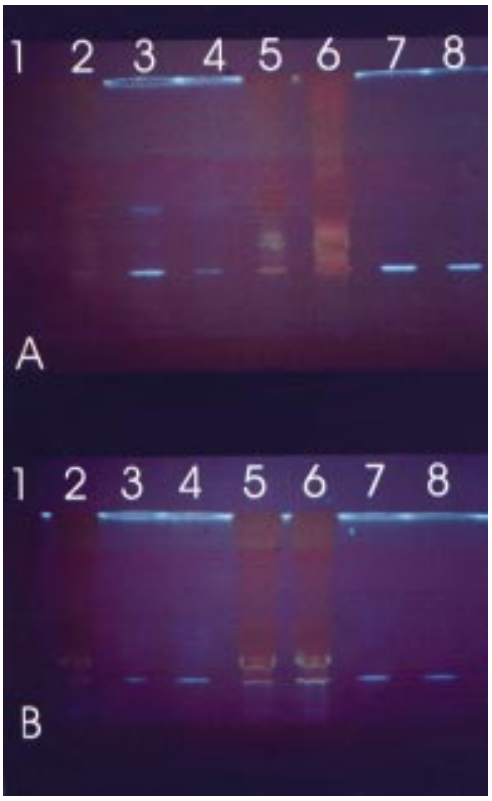

Figure 2 TLC profile of chloroform soluble fluorophores generated by incubating retinal with ROS. Lanes 1 to 4 contain freshly prepared material; lanes 5 to 8 material after 7 days' incubation. Plate (A) contains samples at pH 7, plate (B) samples at pH 4.5. Lanes 1 and 5, retinal + ROS + BSA; lanes 2 and 6, retinal + ROS; lanes 3, 4, 7, and 8, ROS alone. The plates shown are representative of a typical experiment.

generated using intact ROS, the absolute position of spectral peaks did show some small variations, peak intensities were lower, and fluorophores tended to show lower R_f values. In the absence of BSA, these fluorophores appeared more 'orange' but no change in fluorophore mobility was noted (data not shown).

REACTION OF LIPOSOMES WITH RETINOL AND RETINAL

In general, incubation of liposomes with retinal resulted in a pH dependent generation of long wavelength emitting, yellow/orange, fluorophores with lipofuscin-like characteristics (Figs 3 and 4, Table 3). These fluorophores were only observed at pH 7 and their formation was not affected by the inclusion of BSA in the reaction mixture. No long wavelength emitting fluorophores or yellow/orange bands were seen when retinol was used in place of retinal.

Incubated mixtures of liposomes and retinal turned dark yellow/orange after 1 to 2 hours and thereafter no further colour changes occurred. Both excitation and emission spectra of incubated solutions showed characteristics indicative of the presence of lipofuscin-like fluorophores. Fluorophores generated were excited at 350, 400, and 479 nm; emission spectra indicated the presence of long wavelength emitters (416, 588, and 625 nm). Unlike the spectra of retinal/ROS mixtures, where such fluorophores were only minor parts of the overall spectrum, spectra of liposomes/retinal indicated that the long wavelength emitters predominated (Fig 3). Upon extraction and analysis by TLC, nine yellow/orange emitting fluorophores were present (Fig 4, Table 3). Four of these fluorophores (R_f s = 0.38, 0.5, 0.54, 0.73) had similar appearance and chromatographic mobility to fluorophores of lipofuscin (R_f s = 0.36, 0.5, 0.57, 0.71) (Fig 4, Table 3). Fluorophores had formed within 7 days and thereafter no further changes occurred during incubations of up to 42 days. Similar observations were made in the absence of BSA.

No such fluorophores were generated for samples of retinol incubated with liposomes with/without BSA or liposomes incubated alone. Spectral peaks increased in intensity during the incubation period but only small spectral shifts were observed (Table 3). TLC revealed the presence of mainly blue emitting fluorophores (Table 3).

Table 2 R_f values of fluorophores formed during incubation of vitamin A with intact ROS and BSA

	R_f values of bands		
		Incubated	
Substrate mixture	*Fresh*	*pH 4.5*	*pH 7*
ROS	0.45(B),0.55(B),0.6(B/Y), 0.8(B),0.93(B/W)	as fresh	as fresh
BSA	no visible bands	no visible bands	no visible bands
ROS+BSA	0.35(B),0.41(B),0.46(B),0.54(B)	as fresh	as fresh
Retinal	0.58–0.6(O)*	0.6–0.65(P)*	0.6–0.65(O)*, 0.92(P)*
Retinol	0.625–0.675(Y)	0.5–0.6(Y)*	0.42–0.51(Y)*, 0.55–0.75(Y)*
ROS + retinal	0.38(Y), 0.65(Y)	0.38(O/Y), 0.58(Y/O), 0.6(Y/O)	0.38(O),0.45(Y),0.48(O/Y), 0.6(Y),0.74(Y)
BSA + retinal	0.33(Y),0.79(O)	0.26(Y),0.6(P)*	0.35(Y),0.56(P)*,0.85(O)
ROS + BSA + retinal	0.38(Y), 0.65(Y)	0.38(O/Y), 0.58(Y/O), 0.6(Y/O)	0.38(O),0.45(Y),0.48(O/Y), 0.6(Y),0.74(Y)
ROS + retinol	0.45(B),0.55(B/Y),0.6(B), 0.8–0.93(O)*	as fresh	as fresh
BSA + retinol	0.36(B)	0.43(Y), 0.59–0.73(Y),0.76(O)*+smear	0.43(Y), 0.59–0.73(Y), 0.76(O)*+smear
ROS + BSA + retinol	0.43(Y),0.46(B/Y),0.52(O)*	as fresh	as fresh

Letters in parentheses indicate colour of bands (O = orange, B = blue, Y = yellow, P = purple). R_f = distance travelled (band/solvent front). *= non-fluorescent band; ROS = rod outer segments; BSA = bovine serume albumin.

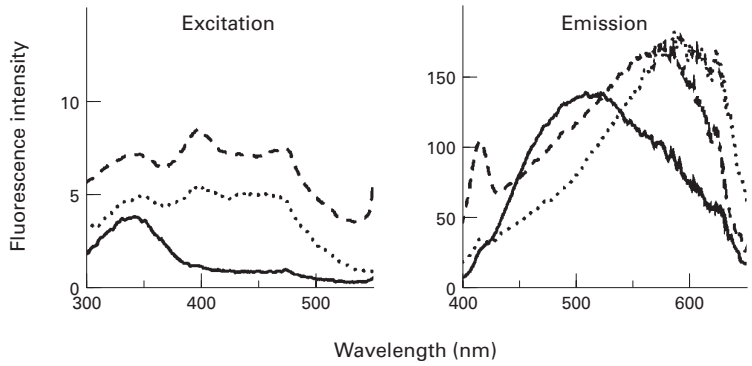

Figure 3 Spectral profile of fluorophores formed by incubating retinal with liposomes. Spectra shown are of a freshly prepared (—) and incubated (. . .) retinal/liposome mixture and are representative of a typical experiment. A similar profile was produced in the presence of BSA. A typical spectral profile of lipofuscin isolated from five 60 to 69 year old donors (– –) is shown for comparison.

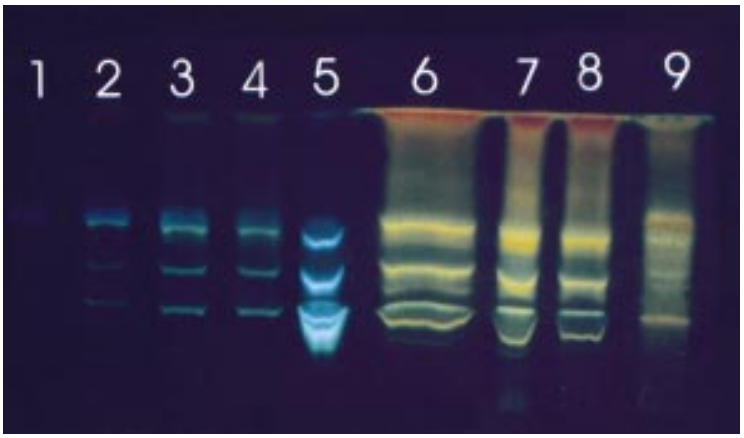

Figure 4 TLC profile of chloroform soluble fluorophores generated by incubating liposomes with retinal. Lanes 1 to 4 contain freshly prepared material; lanes 5 to 8 material after 7 days' incubation. Lanes 1 and 5, liposomes; lanes 2 and 6, liposomes + retinal; lanes 3, 4, 7, and 8, liposomes + retinal + BSA; lane 9, lipofuscin. The plate is representative of a typical experiment.

EFFECT OF REACTANT CONCENTRATION ON FLUOROPHORE FORMATION

Increasing the concentration of retinal in incubated solutions of ROS and BSA resulted in bands appearing more 'orange' and some small variations in fluorophore mobility. However, no new bands were formed and no variations in spectral profiles detected, although peak intensity did increase. Changing the concentrations of BSA or retinol did not change the fluorescence spectra or TLC banding pattern (data not shown).

EFFECT OF LYSOSOMAL ENZYMES ON FLUOROPHORE FORMATION

Incubating the different reaction mixtures with either RPE extract or the lysosomal enriched fraction of bovine liver resulted in only minor changes in the spectral profile and/or fluorophore mobility compared with samples in the absence of lysosomal treatment. However, both peak and band intensities tended to be higher in the absence of extract (data not shown).

Discussion

The original in vitro models used to study lipofuscinogenesis were based on mixtures of lipid peroxidation byproducts incubated with amino acids. Chloroform extracts of the resultant fluorescent compounds were deemed to be photophysically comparable with extracts of lipofuscin.[27] Doubt has since been cast on the suitability of these models for the genesis of ocular lipofuscin. Firstly, the spectral characteristics of ocular lipofuscin measured both in vivo[28] and in vitro[22 29] show a broad emission spectrum from 500 to 750 nm in contrast with the blue emissions reported to arise from reactions of lipid peroxidation byproducts.[27 30] Secondly, spectrophotometer insensitivities have been shown to cause true yellow emitting fluorophores to appear blue when uncorrected spectra are used.[29] Eldred and Katz repeated the conditions of the original experiments using lipids isolated from rat retina and found the resultant fluorophores to differ from ocular lipofuscin in spectral profile (using corrected spectra), solubility, and chromatographic mobility.[31] This absence of lipofuscin-like fluorophores may have been caused by the lack of vitamin A or its derivatives in the reaction mixture.

Our study identifies a key role for retinal in the generation of at least some of the fluorophores of ocular lipofuscin, and is supported by a recent study by Katz *et al*.[32] Although structural characterisation of the fluorophores generated in this study was not

Table 3 R_f values of fluorophores formed during incubation of vitamin A with liposomes and BSA

	R_f values of bands	
Substrate mixture	*Fresh*	*Incubated pH 7*
LIP	0.35(B),0.41(B),0.49(B),0.59(B)	0.11(Y/G),0.19(Y),0.27(B/G),0.29(B/G), 0.32(B), 0.35(B),0.46(B),0.57(B),0.65(B),0.73(B)
BSA	no visible bands	no visible bands
LIP + BSA	0.35(Y),0.48(B),0.57(Y)	0.35(b),0.48(Y),0.57(B),0.86(Y)
Retinal	0.58–0.6(O)*	0.6–0.65(O)*, 0.92(P)*
Retinol	0.625–0.675(Y)	0.42–0.51(Y)*, 0.55–0.75(Y)*
LIP + retinal	0.35(B),0.46(Y),0.59(B)	0.11(Y),0.24(Y),0.27(Y),0.38(O/Y), O.5(O),0.54(O),0.73(O),0.95(O),0.97(O)
BSA + retinal	0.33(Y),0.79(O)	0.35(Y),0.56(P)*,0.85(O)
LIP + BSA + retinal	0.35(B),0.46(Y),0.59(B)	0.11(Y),0.24(Y),0.27(Y),0.38(O/Y),O.5(O), 0.54(O),0.73(O),0.95(O),0.97(O)
LIP + retinol	0.35(B),0.46(Y),0.59(B)	0.32(B),0.35(B),0.46(B),0.57(B),0.65(B),0.73(B)
BSA + retinol	0.36(B)	0.43(Y), 0.59–0.73(Y),0.76(O)*+smear
LIP + BSA + retinol	0.35(B),0.48(Y),0.59(B)	0.37(B),0.48(Y),0.57(B),0.86(Y)

Letters in parentheses indicate colour of bands (O = orange, B = blue, Y = yellow, P = purple). R_f = distance travelled (band/solvent front). *= non-fluorescent band; LIP = liposomes; BSA = bovine serume albumin.

undertaken, speculation as to their structural nature can be made. Fluorophores were only formed in the presence of retinal and lipid, indicating they were a reaction product of these two substrates. Two key factors appear to be necessary for fluorophore formation: (1) bonding between retinal and lipid (fluorophores were not formed when substrates were incubated alone) and (2) the aldehyde group of retinal (when retinol was used in place of retinal, fluorophore formation did not occur). The retinal/lipid bond may involve a condensation reaction between the carbonyl group of retinal and lipid oxidation products or a Schiff's base with amino lipids. Retinal can also become oxidised to generate a variety of carbonyl containing groups, providing other potential reactants.[25 26] The lack of fluorophores formed with retinol may be due to (1) a decreased susceptibility of the alcoholic group to oxidation, (2) the non-involvement of vitamin A oxidation products in fluorophore formation, and/or (3) an absolute requirement for retinal.

It has been suggested that long wavelength emitting fluorophores must consist of a highly conjugated system of double bonds in which absorbed irradiant energy can be dissipated before emission.[33] The reaction products of polyunsaturated fatty acids and retinal may provide such a conjugated system. Variations in spectral profiles and fluorophore mobility in mixtures of retinal incubated with the different lipid substrates may reflect variations in the types of fatty acid present and the degree of unsaturation. The total fatty acid content of the bovine retina has been reported to contain 50% unsaturates,[34] with the major unsaturated fatty acid being docosahexanoate (22:6ω3), while the liposomes contain 54% unsaturates with octadecanoic acid (18:1) as the major unsaturated species.

The observation that fewer fluorophores were generated using intact ROS compared with liposomes may reflect a decreased accessibility of retinal to reactive lipid components or susceptibility of the lipid to oxidation. Intact ROS were in the form of an intact bilayer so may not have been able to react with vitamin A or protein to generate the chloroform soluble fluorophores characteristic of lipofuscin. Breakdown of this bilayer by extraction may have destroyed or removed groups capable of fluorophore formation. In vivo, the lipid bilayer is digested by the action of lysosomal enzymes.

Attempts to break down the ROS to a more reactive form by the action of lysosomal enzymes had no effect on the fluorophore formation, suggesting that enzymes were either (1) not sufficiently concentrated or (2) not involved in fluorophore formation. The latter is most likely since enzyme levels used in these experiments were either comparable with or higher than those reported in vivo or in cultured RPE cells.[35–37]

It was interesting that long wavelength emitting fluorophores, similar to lipofuscin, only formed at pH 7. This may be due to the higher rates of lipid oxidation at acid pH altering the balance of reactants such that lipid/lipid rather than lipid/retinal interactions are favoured. In vivo, lipofuscin resides within the acid environment of the lysosomal vacuome, suggesting that at least initially, fluorophore formation must occur external to this environment. Such reactions may occur in the ingested phagosome before fusion with acidified vesicles containing lysosomal enzymes.[38] Alternatively, the presence of less drastic oxidising conditions in vivo may allow fluorophore formation at pH 4.5.

Protein was not involved in the generation of lipofuscin-like fluorophores in this model system. However, it is possible that the incubation time was insufficient to allow the formation of fluorophores involving vitamin A/protein or lipid/protein adducts. A further possibility could be that fluorophore formation is dependent on the amino acid content of BSA and ROS proteins; the latter contain high levels of lysine and arginine residues which have a free nitrogen group and are therefore capable of forming Schiff bases with carbonyl groups of oxidised lipid and retinal.

In conclusion, these results suggest some of the chloroform soluble fluorophores of lipofuscin may be formed as a direct reaction product of retinal and lipid. Further study is required to analyse chloroform insoluble fluorophores known to be present at the interface[31] and non-fluorescent material present in lipofuscin.

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