Total Luminescence Spectroscopy of Fluorescence Changes during Aging in *Caenorhabditis elegans*†

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ABSTRACT: Total luminescence spectroscopy was employed to characterize and quantitate age-related changes in fluorescence in the nematode *Caenorhabditis elegans*, an established model for aging research. The excitation wavelength was varied between 250 and 590 nm in 10-nm increments. At each excitation wavelength, the emission wavelength was varied between 300 and 600 nm. Contour plots of corrected spectra were made. All fluorescence increased severalfold with age, except for that ascribed to tryptophan of soluble protein fractions. This general increase included fluorescence due to flavins, which is not expected to increase with age but has previously been observed to do so in this species. Blue emission peaks that approximated Schiff base product fluorescence were detected in whole aqueous homogenates, chloroform/methanol extracts, and detergent-cleaned cuticle preparations. Age-related increases in emission intensities of these peaks were demonstrated in aqueous homogenates and isolated cuticles. Cuticle preparations, known to be rich in collagenous protein, exhibited a fluorescence peak that approximated the recently described pyridinoline cross-link of vertebrate collagen. This peak, as well as the entire cuticle emission spectrum between 300 and 500 nm, increased dramatically with age. A fluorescence peak tentatively identified as cuticle tyrosine, characteristic of collagenous protein, also increased in older worms. The effectiveness of the spectroscopic technique in distinguishing individual fluorescence peaks in complex mixtures was demonstrated, and the potential of the nematode cuticle for age-altered collagen studies was identified.

One characteristic of aging cells that has been observed throughout the metazoan phyla is the accumulation of fluorescent pigments. Though the functions and the subcellular origins of these pigments remain obscure, their association with membranous organelles has led to speculation that membrane alteration may be implicated in their formation. The production of free radicals and lipid peroxides with subsequent free radical chain reactions is thought to produce membrane damage through cross-linking of proteins and lipids (Leibovitz & Siegel, 1980). Manifestations of this damage involve disruption of cellular ultrastructure, reduction of enzyme activities, and accumulation of fluorescent "age pigments".

Nematodes have been much used as model systems for aging studies. Among the advantages of the nematode model are the short life span, the ease of laboratory culture, and the aspects of nematode aging that appear to parallel events associated with mammalian aging (Gershon, 1970; Zuckerman & Himmelhoch, 1980).

Ultrastructural study of aged *Caenorhabditis briggsae* revealed an age-dependent accumulation of pigment granules in the degenerating intestinal epithelium of this nematode (Epstein et al., 1972). *Caenorhabditis elegans* has become the standard experimental nematode species since Brenner (1974) demonstrated its advantages for genetic studies (Zuckerman, 1980). Klass (1977) demonstrated an age-related increase in fluorescence in aqueous homogenates and chloroform/methanol extracts of *C. elegans*. Excitation and emission wavelengths of this fluorescent material resembled that of lipofuscin age pigment (Fletcher et al., 1973).

Since fluorescent pigment accumulation is commonly used as an index of physiological age, refinement of measurement techniques is desirable to increase the utility of this index. Unfortunately, a number of fluorescent biomolecules approximate age pigments in excitation and emission wavelengths and could interfere with the accuracy of their fluorometric determinations. Total luminescence spectroscopy (Giering & Hornig, 1977) was employed in this study to characterize more completely the fluorescent materials in *C. elegans* and their changes with age.

Materials and Methods

Synchronous cultures of *C. elegans* were grown monoxenically at 20 °C on agar plates with *Escherichia coli* lawns (Brenner, 1974). Synchrony was established by inoculating plates with L_1 larvae from stock N_2 cultures that passed through a Nitex nylon filter (10-μm pores). During the egg laying phase of the life cycle (days 3–8), larval contaminants were removed by daily filtration through a 400-mesh (400 weaves/in.) stainless steel filter. This filter retained adults while passing larval stages. Subsequent to filtration, aliquots of worm samples were heat killed and measured to monitor their growth in culture as well as to detect larval contaminants that were retained by the 400-mesh filter. Worms were returned to fresh plates, and larval contaminants were killed with a hot needle. For fluorometric assays, worms were recovered at 4 days (young adults) and at 12 days (old adults), washed in distilled, deionized water, and frozen in liquid nitrogen until used.

Aqueous homogenates were prepared by suspending aliquots of frozen worms in 3 mL of deionized water and subjecting them to repeated freeze–thaw cycles, followed by sonication. Worm disruption was confirmed by microscopic examination.

For identification of the fluorescence associated with large particulate material in these homogenates, it was pelleted by centrifugation for 10 min at 2000*g* and washed twice in 2 mL of deionized water. Resultant pellets were resuspended by stirring in 3 mL of deionized water and assayed fluorometrically for comparison with the whole aqueous homogenates.

Lipid fractions were obtained by vigorously agitating worm samples for 2 min at room temperature in 5 mL of chloroform/methanol (2:1). Both the chloroform and methanol were glass distilled to reduce fluorescent contaminants, and the glassware employed throughout all experimental procedures

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was cleaned in acid ethanol prior to use. Chloroform/methanol-insoluble material was pelleted by centrifugation and assayed for Lowry protein after drying under a stream of N_2 gas. The chloroform/methanol supernatants were washed twice with equal volumes of deionized water to remove flavin contaminants (Fletcher et al., 1973). Centrifugation of the chloroform/methanol/water mixture separated the chloroform and water/methanol fractions. Both the washed chloroform fraction and the first water/methanol fraction were analyzed fluorometrically.

Purified cuticle fractions were prepared by incubation of ruptured worm samples at 37 °C in 1% sodium dodecyl sulfate ($NaDodSO_4$) according to the technique of Leushner et al. (1979). $NaDodSO_4$-cleaned cuticles were collected by centrifugation and washed twice in deionized water. For fluorometric analysis, the washed cuticle fragments were resuspended in 3 mL of deionized water.

For reduction of inner filter effects on fluorescence intensity, all samples were diluted so that the optical density (OD) for a 1-cm path at the excitation wavelength of primary interest (360 nm) was less than 0.05. The OD at longer wavelengths decreased because turbidity dominated absorption. At shorter wavelengths the OD increased, but even in the worst case (whole homogenate containing nucleic acids) the protein peak (280–290 nm) OD did not exceed 0.12. The maximum OD (250–260 nm) in the whole homogenates ranged from 0.14 to 0.17. Thus, fluorescence intensities observed at extremely short excitation wavelengths may be reduced. Dilution tests showed no interference with linearity of the protein fluorescence intensity at these concentrations. Similarly, dilutions of the various fractions assayed confirmed linearity between fluorescence intensity and concentration for all major peaks.

Samples from both age groups prepared as described above were subjected to total luminescence spectroscopic analysis. Fluorescence intensity was measured at 10-nm intervals (8-nm bandwidth) with excitation wavelengths (250–590 nm) and emission wavelengths (300–600 nm) varying to yield a two-dimensional array of data points. An SLM photon-counting spectrofluorometer (SLM Instruments, Inc., Urbana-Champaign, IL) controlled by a Hewlett-Packard 9815S computer was employed to collect data. A 1-cm^2 quartz cuvette, with stirring and temperature controlled at 20 °C, was used to hold the sample. Spectral data arrays were formatted via a central computer by using Calcomp programs to generate linear contour plots in which tics indicate the high-intensity sides. Contours were plotted only in the region bounded by the points 250,300, 280,300, 580,600, 330,600, and 250,490 in order to avoid the relatively high residual intensities from first- and second-order scattering.

All spectra were excitation and emission corrected with a rhodamine quantum counter and a MgO smoked screen. Further correction for solvent fluorescence and Raman scattering was accomplished by subtracting spectra of solvent blanks from all data spectra. The background from scattered light leaking through the monochrometers was removed by subtraction of a solvent-corrected spectrum obtained from glycogen in water.

Fluorescence spectra were adjusted to compensate for protein concentration to allow computer averaging of spectra from replicate experiments and to permit comparisons among sample groups when appropriate. Protein determinations were performed according to the method of Lowry et al. (1951) by using comparably treated bovine serum albumin standards. Insoluble protein was solubilized in hot 1 M NaOH. The various fractions were normalized to the protein in the samples

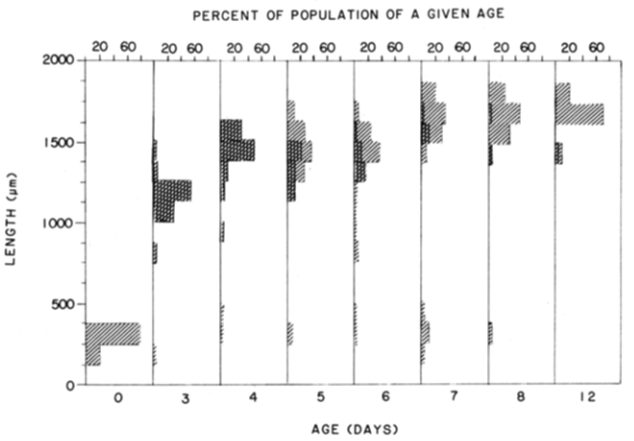

FIGURE 1: Growth in synchronous culture. For each age, the histogram indicates the distribution of the total population by length. Cross-hatching indicates worms containing eggs. Age is measured from the L_1 stage.

used for fluorescence spectra with the following exceptions: (1) the chloroform-soluble fraction and the water/methanol wash were normalized to protein that precipitated from the chloroform/methanol wash; (2) the particulate fraction was normalized to total protein in the homogenate from which it was obtained.

Statistical analysis of numerical data employed *t* test comparisons of mean relative fluorescence intensities and relative intensity ratios. Means were considered significantly different if the probability that they were from the same population was less than 5% ($P = <0.05$). Duplicate extractions were performed on each of two worm cultures of each age group. Since statistical analysis showed no differences between duplicate cultures of the same age, data from all four extractions were pooled for a given age.

Several of the fluorescence peaks can be tentatively identified by comparison of the fluorescence spectra with those of known compounds that are likely to be present in relative abundance in particular fractions. For convenience these peaks will be referred to by these most probable identities, recognizing that rigorous identification has not been performed.

Results

Synchronous Cultures. Larval worms that were used to inoculate synchronous cultures showed a mean length of 326 ± 63 (SD) μm and increased in size to 1693 ± 84 (SD) μm by day 12 (Figure 1). The percent of egg-bearing worms, as evidenced by the in utero presence of shelled embryonated eggs, peaked at 100% at days 3 and 4 and decreased to less than 5% by day 8. Second-generation larval contaminants were less than 10% of the worms present. Because of their relatively small size, this contamination represents less than 0.1% of the total worm volume. Survival curves were not determined, but the work of Klass (1977) suggests approximately 50% survival by day 12.

Aqueous Homogenates. Average spectra obtained from replicate aqueous homogenates of 4-day and 12-day worm samples revealed four major emission peaks (Figure 2). Excitation and emission wavelengths for fluorescence maxima will be presented in the format (excitation wavelength in nanometers; emission wavelength in nanometers). Peak P (280–290; 320–340) was tentatively identified as protein tryptophan fluorescence by comparison with spectra of aqueous bovine serum albumin (BSA) and tryptophan standards. Qualitatively, the wavelength coordinates and general contours of this peak were similar in 4-day and 12-day samples.

Table I: Age Differences in Whole Aqueous Homogenate Fluorescence[a]

peak:	F_1 (flavin)			F_2 (flavin)			B (blue)			P (protein tryptophan)		
λ_{ex}; λ_{em}:	440–450; 540–560			370–380; 540–560			330–350; 430–460			280–290; 320–340		
age group (days)	4	12	12/4	4	12	12/4	4	12	12/4	4	12	12/4
mean intensity	2.62 ± 1.10	6.47 ± 0.52	2.5	1.06 ± 0.21	4.04 ± 0.05	3.8	1.16 ± 0.52	6.38 ± 1.10	5.5	7.07 ± 1.60	8.16 ± 0.49	1.2
P value	<0.01			<0.01			<0.01			>0.05		
mean ratio	0.37 ± 0.12	0.79 ± 0.02	2.1	0.16 ± 0.04	0.49 ± 0.02	3.1	0.16 ± 0.04	0.79 ± 0.08	4.9			
P value	<0.01			<0.01			<0.01					

[a] Mean intensity is expressed as relative fluorescence per unit protein concentration ± standard deviation ($n = 4$). Mean ratio is the measured intensity of each peak relative to protein peak intensity ± standard deviation ($n = 4$).

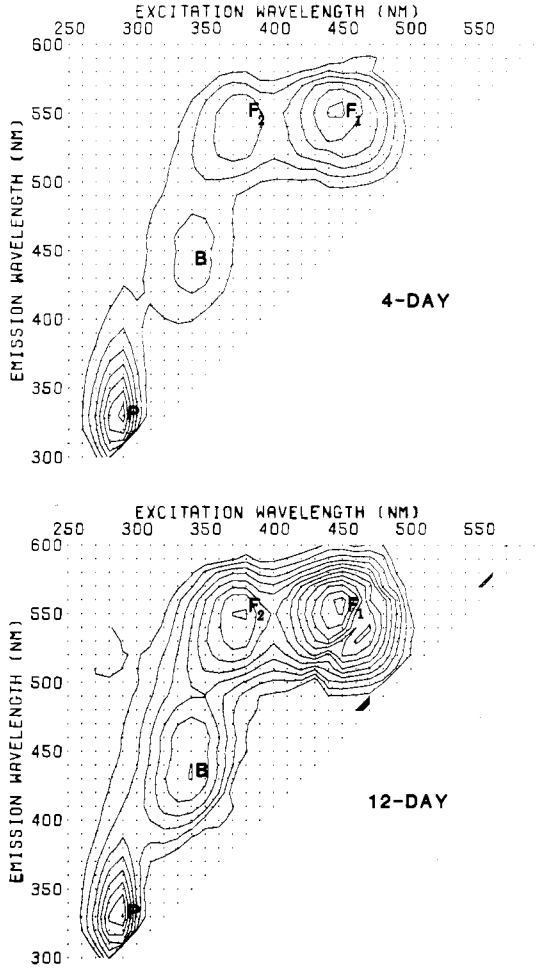

FIGURE 2: Spectra of 4-day and 12-day whole aqueous homogenate fluorescence: (F_1 and F_2) flavin peaks; (B) blue fluorescence; (P) protein tryptophan peak. Contour intervals for both spectra were 10.

Quantitatively, the protein fluorescence peak showed no significant difference between the two age groups when normalized to Lowry protein (Table I).

Comparison of Lowry protein assays on soluble and insoluble fractions of whole homogenates revealed that most of the total protein existed as water-soluble protein (82% in 12-day samples and 92% in 4-day samples). Statistical comparisons of the ratios of insoluble to total protein from 4-day and 12-day homogenates showed a significant increase in insoluble protein with older worms. The 12-day worm samples had 2.3 times more insoluble protein than the younger worms.

Probable flavin fluorescence, a common contaminant of age pigment extractions, is represented by peaks F_1 (400–450; 540–560) and F_2 (370–380; 540–560) as indicated by comparison with flavin adenine dinucleotide (FAD) standards. A third flavin peak (270; 540–560) was present in the raw spectra. Because of its proximity to the residual second-order scatter, it was outside the areas plotted for the spectra presented here.

As with protein fluorescence, the flavin peaks were qualitatively similar in both age groups. The F_1 flavin peak extended across the Raman scatter line so that residual errors in solvent correction probably produced the shoulder (460–470; 530–540).

Quantitatively, flavin fluorescence increased significantly with age in the aqueous homogenates. The F_1 peak increased by a factor of 2.5 when normalized to Lowry protein. Similarly, the F_2 peak increased by a factor of 3.8 (Table I). Internal normalization by comparing the ratios of flavin peak intensities to protein peak intensities showed slightly lower age-related increases in both flavin peaks. The greater increase in F_2 with age might be due to overlap with the blue peak or might indicate changing ratios of various flavin species.

The peak of blue fluorescence (330–350; 430–460) could not be identified because it corresponds to many fluorescent biomolecules, including Schiff base products. Regardless of its identity, it showed a significant increase in intensity with age that was greater in magnitude than either of the flavin peaks (Table I). This blue fluorescence was over 5-fold greater in intensity in the 12-day samples than in the 4-day controls when normalized to Lowry protein. Internal normalization by comparing the peak intensity relative to protein fluorescence confirmed this age-dependent increase.

Chloroform/Methanol Extracts. Washed chloroform/methanol extracts from worms of the two age groups revealed only one major peak (330–350; 390–420) with a pronounced shoulder into longer emission wavelengths (Figure 3). The shoulder at (340–350; 460) may represent a separate emission peak. Again, identification of this fluorescence was not possible.

Spectra from the first water/methanol fraction (Figure 4), when compared qualitatively with the chloroform fractions, showed that flavin contaminants can be effectively removed by water washing the chloroform/methanol extract. Interestingly, the blue fluorescence area that was apparent in the chloroform fraction appeared as well in the water/methanol phase. Excitation wavelengths of this blue fluorescence were identical in both fractions, but the emission wavelengths of the primary peak at λ_{em} = 390–420 nm in the chloroform phase shifted to 420–440 nm in the water/methanol fraction. The longer emission wavelength shoulder remained at the same wavelength coordinates in both fractions.

Comparisons between the 4-day and 12-day groups showed no qualitative differences as a function of age in either chloroform-soluble or water/methanol-soluble fluorescence. Consistent with recent reports on the effectiveness of chloroform/methanol in age pigment extraction (see Discussion), this procedure proved too variable for quantitative comparison. Average spectra of the water/methanol fractions suggested

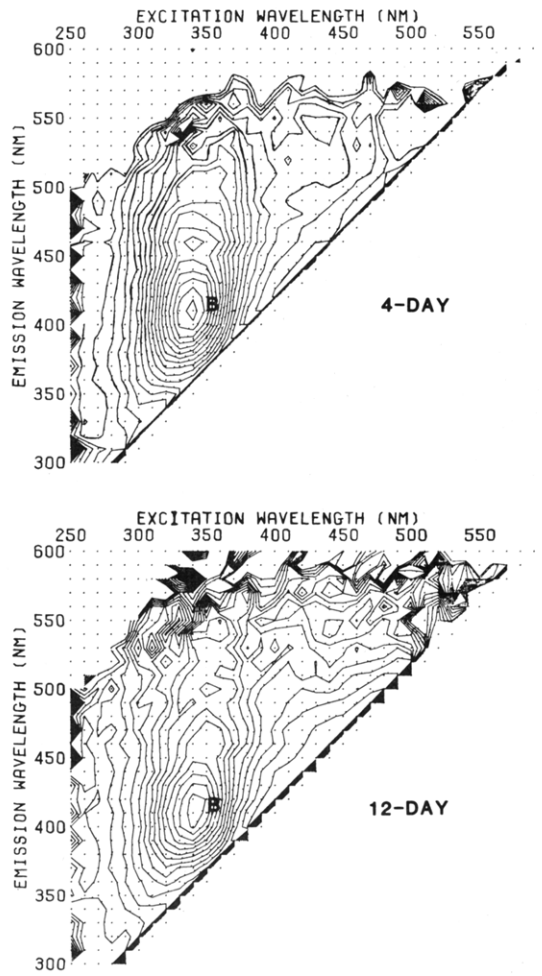

FIGURE 3: Spectra of 4-day and 12-day chloroform-soluble fluorescence: (B) blue fluorescence peak. Contour intervals for both spectra were 10.

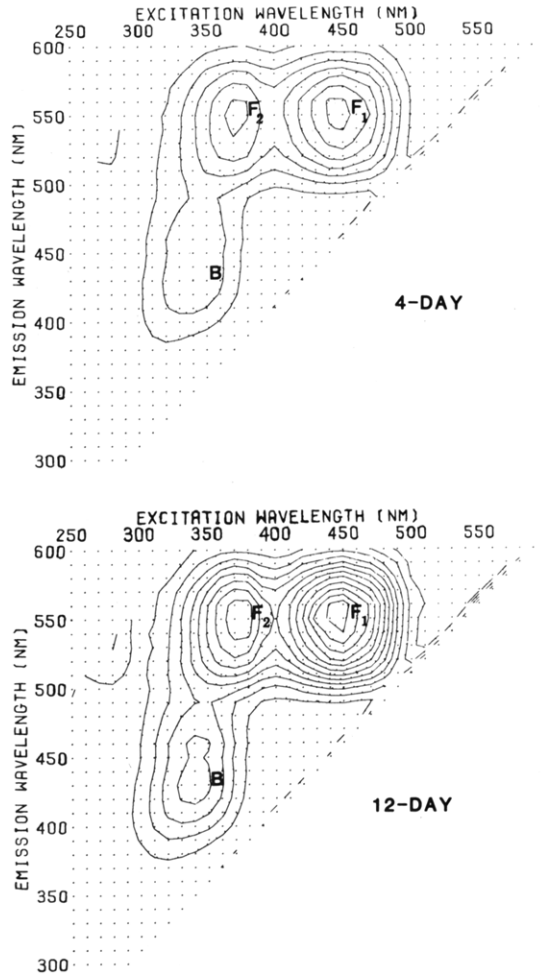

FIGURE 4: Spectra of 4-day and 12-day fluorescence from the first water/methanol fraction: (F_1 and F_2) flavin peaks; (B) blue fluorescence. Contour intervals for both spectra were 50.

an age-related increase in both flavin and blue fluorescence, but poor reproducibility obscured any statistical significance.

Large Particulates. So that large particulate fluorescence could be differentiated from the total fluorescence of whole aqueous homogenates, water-washed, insoluble pellets were recovered by centrifugation. Spectra obtained from this insoluble material (Figure 5) showed one major peak of protein fluorescence (280–290; 320–340) and an area of weak fluorescence (340–370; 450–470). The protein peak was qualitatively similar to that of the whole homogenates with the exception that the range of emission wavelengths shouldered upward toward 500 nm. This was suggestive of a more complex emission pattern than that of the soluble protein predominating in whole homogenates.

The area of weak fluorescence (S) approximated that of Schiff bases in wavelength coordinates. This area was not apparent in previously described preparations and was too weak to demonstrate any change with age (Table II). The more intense blue fluorescence peak apparently obscured the weaker S area in whole homogenates of both age groups. More importantly, both the flavin peaks and the blue fluorescence of whole homogenates were eliminated by simple water washing. Protein emission intensity increased nearly 4-fold during aging.

$NaDodSO_4$-Cleaned Cuticles. Since the crude water washing technique was too variable for quantitative comparison of the S regions between age groups, $NaDodSO_4$ cuticle isolation was employed to increase reproducibility and produce a more defined sample. Average spectra of $NaDodSO_4$-cleaned cuticles (Figure 6) revealed four major fluorescence

Table II: Age Differences in Insoluble Particulate Fluorescence[a]

peak:	S (?)			P (protein)		
λ_{ex}; λ_{em}:	340–370; 450–470			280–290; 320–340		
age group (days)	4	12	12/4	4	12	12/4
mean intensity	0.02 ± 0.03	0.05 ± 0.03	2.5	0.11 ± 0.03	0.42 ± 0.05	3.8
P value	>0.05			<0.01		
mean ratio	0.22 ± 0.22	0.14 ± 0.09	0.6			
P value	>0.05					

[a] Mean intensity is expressed as relative fluorescence per unit protein concentration ± standard deviation ($n = 4$). Mean ratio is the measured intensity of each peak relative to protein peak intensity ± standard deviation ($n = 4$).

areas that were similar in both age groups. The possible complexity of the protein peak that was suggested by the particulate fluorescence spectra was confirmed by cuticle purification. The protein peak was replaced by one at shorter wave lengths (280; 310–320) characteristic of tyrosine rather than tryptophan. The long-wavelength emission shoulder of the particulate protein peak appeared as a separate peak (300; 400–420) in purified cuticles. This fluorescence (peak A) was not apparent in whole homogenates.

The area of presumed Schiff base fluorescence (340–370; 450–470) showed two separate peaks (S_1 and S_2) in the averaged 12-day spectra that did not appear in the young worm cuticles. A third peak (350–360; 490–500), designated as peak X, was arbitrarily considered separately from Schiff base

Table III: Age Differences in $NaDodSO_4$-Cleaned Cuticle Fluorescence[a]

peak: λ_{ex}; λ_{em}:	A (?) 300; 400–420			S_1 (?) 370; 460–470			S_2 (?) 340–350; 450–460			X (?) 340–360; 490–500			P (protein tyrosine) 280; 310–320		
age group (days)	4	12	12/4	4	12	12/4	4	12	12/4	4	12	12/4	4	12	12/4
mean intensity	0.20 ± 0.08	2.59 ± 0.62	13	0.03 ± 0.04	2.85 ± 0.66	95	0.06 ± 0.08	2.02 ± 0.67	34	0.04 ± 0.04	1.54 ± 0.35	38	0.13 ± 0.07	2.03 ± 0.45	16
P value	<0.01			<0.01			<0.01			<0.01			<0.01		

[a] Mean intensity is expressed as relative fluorescence per unit protein concentration ± standard deviation ($n = 4$).

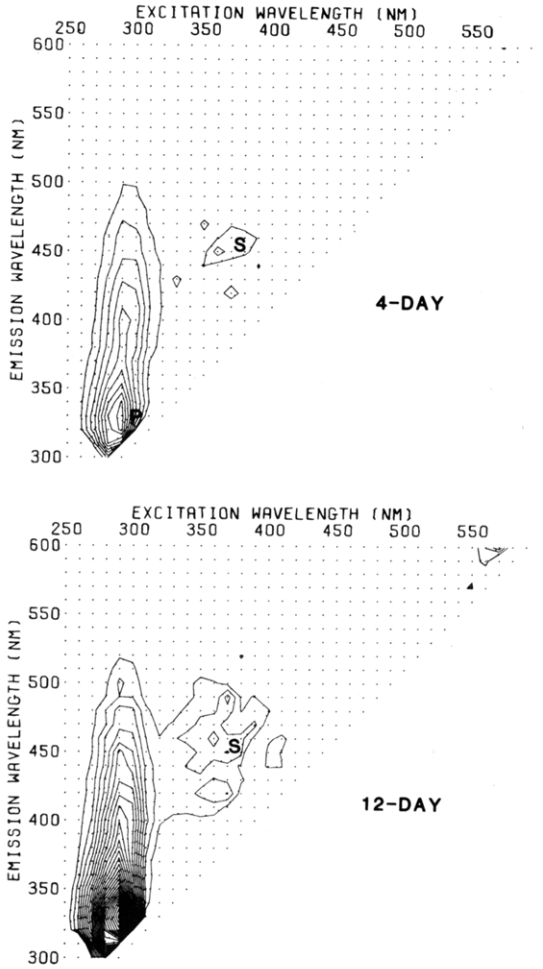

FIGURE 5: Spectra of 4-day and 12-day particulate fluorescence: (P) protein tryptophan peak; (S) area of presumed Schiff base fluorescence. Contour intervals for both spectra were 2.

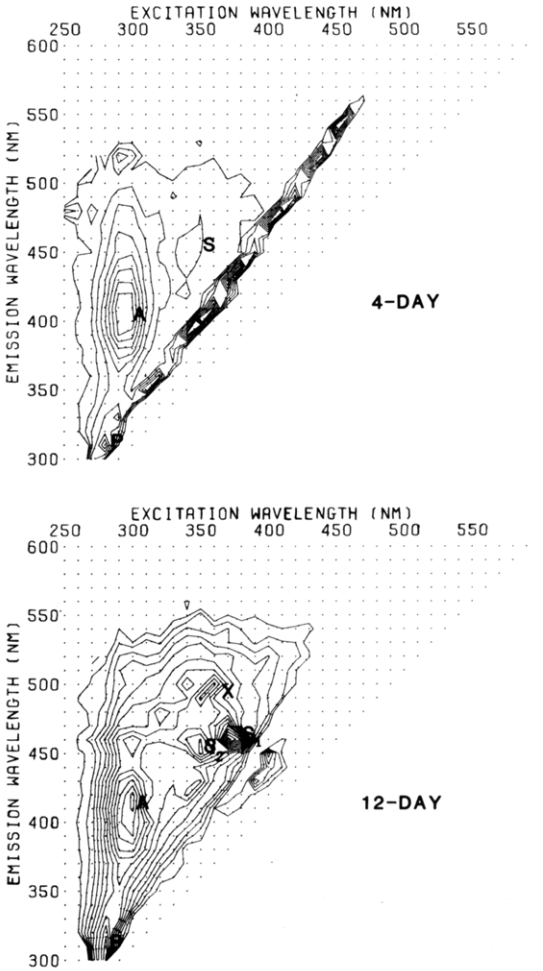

FIGURE 6: Spectra of 4-day and 12-day $NaDodSO_4$-cleaned cuticle fluorescence: (S, S_1, and S_2) possible Schiff base fluorescence; (X) unidentified peak; (A) pyridinoline-like peak; (P) protein tyrosine fluorescence. Because of the magnitude of age differences in intensities, the 4-day spectrum contour interval was 2 and the 12-day spectrum contour interval was 20.

fluorescence because the emission wavelength range was longer than that usually associated with Schiff bases. The peak was not identified. Normalization of the fluoresence intensities to Lowry protein for quantitative comparisons between age groups showed large age-dependent increases in all fluorescence peaks including protein tyrosine fluorescence (Table III).

Discussion

A major finding of this study was that practically all fluorescence increases severalfold with age in *C. elegans*. This was particularly surprising with flavin fluorescence, which is well-defined and would not normally be expected to increase with age. The emission wavelengths (450–580) reported by Klass (1977) for age-dependent fluoresence in *C. elegans* homogenates suggest that flavin fluorescence was being monitored in that instance. Since almost all fluorescence increases with age, an important question to consider is whether the normalization was accurate and appropriate. The accuracy of the Lowry protein measurements was supported by the observation that protein tryptophan fluorescence of homogenates did not change with age when normalized to Lowry protein. In the case of isolated cuticles, the tyrosine fluorescence changed markedly (16-fold) with age when normalized to Lowry protein. From incidental observations made during sample preparation, it was clear that Lowry estimate was much closer to the amount of material present than the tyrosine fluorescence would indicate. Thus, we are satisfied that the Lowry protein measurements are accurate and that the age-related increase in tyrosine fluorescence is real.

This leaves the question of the appropriateness of normalizing to protein. It is not obvious what the appropriate factor for normalization should be in aging studies. Volume, wet weight, dry weight, and protein are all commonly used,

with the implicit assumption that it makes no difference because these parameters should have a fixed relation to one another independent of age. Klass (1977) normalized to volume and dry weight. Both his methods indicated the same increase in fluorescence. Although his studies are not directly comparable, due to different culture conditions and undefined fluorometer characteristics, they are consistent with the observations reported here in that fluorescence increased during aging. It does not appear that inappropriate normalization can explain the general increase in fluorescence. The logical conclusion is that flavin fluorescence increases with age along with that of other materials that fluoresce in the blue region.

Fletcher et al. (1973) reported a wavelength range of age-dependent fluorescent pigments (340–370; 420–470). Related studies have employed fluorescence spectroscopy to explore the effects of lipid oxidation products on biological systems. Reaction of lipid oxidation products with free amino acids yields fluorescence chromophores within the wavelength range of age pigment (Chio & Tappel, 1969; Shimasaki et al., 1977). The phospholipid phosphatidylethanolamine also yields similar fluorescence spectra when reacted with lipid oxidation products (Dillard & Tappel, 1973). Similarly, the peroxidation of membranous subcellular organelles such as mitochondria, microsomes, and lysosomes yields fluorescent products comparable to age pigment (Chio et al., 1969; Dillard & Tappel, 1971). From these findings, it is apparent that a variety of biological molecules can provide the primary amines necessary for production of the fluorescent chromophores of aging. Consequently, the composition of age pigments should be quite heterogeneous and difficult to identify conclusively.

The blue fluorescence peak that characterized whole aqueous homogenates of *C. elegans* (330–350; 430–460) did occur at the low end of the wavelength range previously published for age pigment and may represent conjugate Schiff base products. Unfortunately, the blue peak is in a region where one finds the fluorescence of many biomolecules in addition to age pigment. Two specific examples are known to be present in *C. elegans*, reduced nicotinamide adenine dinucleotide (NAD) (350; 450) and anthranilic acid (320; 400), the latter demonstrated by Babu (1974). Thus, the increase in fluorescence intensity in this region cannot at this time be ascribed to age pigment exclusively. Detailed chemical fractionation will probably be necessary to resolve the compounds contributing to the increase in fluorescence in this region.

Pending chemical analysis, certain characteristics of the blue fluorescent material can be deduced from the present experimental protocol. The peak of blue fluorescence that appeared in washed chloroform extracts had the same excitation wavelength and similar "bilobed" contour topography as the blue peak of aqueous homogenates, suggesting a comparable composition. The observation that a similar blue peak appeared in both the washed chloroform and water/methanol fractions suggests a degree of solubility in both polar and nonpolar solvents. Spectra of particulate fluorescence after water washing did not show the blue peak. Thus, should the material be granular, as previously described for age pigments, it is sufficiently small to escape sedimentation at 2000*g* and is apparently not associated tightly with cuticle fragments or their adherent cytoplasmic debris. Though the emission wavelengths of the chloroform-soluble blue peak in this study were slightly shorter than those reported by Klass (1977) for aged *C. elegans* extracts, they were in agreement with the data of Buecher & Hansen (1974) for *Panagrellus redivivus* and mouse heart extracts. The *C. elegans* model apparently accumulates blue fluorescent materials with age comparable to mammalian systems.

Considering the potential heterogeneity of the blue fluorescence, it is reasonable to expect variability in its extraction. Bieri et al. (1980) found chloroform/methanol extracts to be of little value for quantitative studies of lipofuscin formation in rat uteri when compared with histological evaluation. Their work revealed more fluorescence (350; 440) in the water phase of chloroform/methanol extracts than in the chloroform phase. Allowing for differences in contour intervals of averaged spectra from water/methanol and chloroform phases (Figures 3 and 4), the present study also shows greater blue fluorescence in the water phase. Variability in the chloroform/methanol extracts in this study also made useful quantitative comparisons between age groups impossible. However, qualitative comparisons of spectra from chloroform and water phases were possible since the peaks and their wavelength coordinates did not vary across replicate experiments. Within these limitations, this work did confirm the effectiveness of water washing in removal of flavin contaminants from chloroform extracts and the ineffectiveness of chloroform/methanol in quantitative pigment extraction.

Sedimentation of particulates provided an additional source of age-dependent fluorescence. Microscopic examination of pelleted material revealed a predominance of cuticle fragments, and spectrofluorometric assay showed a large peak of protein fluorescence with a low-intensity area of possible Schiff base fluorescence. Since nematode cuticles are primarily collagen protein, $NaDodSO_4$ purification of cuticles was used to explore the potential of the *C. elegans* model for studies of age-related alteration of collagen.

Electron microscopy has revealed morphological changes in the nematode cuticle that correlate with age. Zuckerman et al. (1973) described an age-dependent accumulation of electron-dense, granular material in the medial layer of the *C. briggsae* cuticle. Scanning electron microscopy has demonstrated a marked wrinkling of the *C. briggsae* cuticle with aging (Högger et al., 1977). Collagenous protein of mammals is known to decrease in elasticity and solubility with age and to increase in fluorescence (Puelo & Sobel, 1972). These changes in physical properties are thought to be a function of increased intermolecular cross-linking, beginning initially with the formation of Schiff base products. Fluorescent Schiff bases may originate from endogenous aldehydes arising from modified amino acids (lysine and hydroxylysine), in which ϵ-amino groups are converted to aldehydes (Tanzer, 1973). Alternatively, reducing sugars can react with ϵ-amino groups of lysine residues to generate fluorescent (360; 420–430) cross-links (Monnier & Cerami, 1981). Finally, free radical initiated lipid peroxidation products can form fluorescent cross-links with primary amines of proteins (Sundholm et al., 1978). Of these three mechanisms, only the reducing sugar aldimine formation does not involve free oxygen.

Since the nematode cuticle serves as the major respiratory surface, it is reasonable to assume its susceptibility to oxygen damage. Regardless of the actual mechanism of damage, the low turnover rates of collagenous proteins should facilitate the accumulation of these fluorescent products with age. In this study, dramatic age-related changes in fluorescence occurred with $NaDodSO_4$-cleaned cuticle fractions, and the proportion of water-insoluble protein in worm homogenates more than doubled with older samples. Both of these observations are consistent with age-related changes in collagenous protein.

Spectra of $NaDodSO_4$-purified cuticles from 12-day samples were consistently greater in intensity over the entire emission

range (300–500 nm) when normalized to Lowry protein and compared to 4-day controls. The blue peak as well as flavin fluorescence that characterized aqueous homogenates were eliminated by $NaDodSO_4$ cleaning.

Protein fluorescence that was the major component of spectra from particulate material appeared as two separate peaks in $NaDodSO_4$-cleaned cuticles. The lower emission peak was characteristic of tyrosine fluorescence, which is to be expected with protein high in collagen content. Tryptophan fluorescence was eliminated by $NaDodSO_4$ purification. The longer emission peak (300; 400–410) corresponded closely in excitation and emission wavelengths to the fluorescent, nonreducible cross-linked compound isolated from bovine achilles tendon collagen (Fujimoto & Moriguchi, 1978). These authors reported uncorrected excitation and emission maxima at 295 and 395 nm, respectively, for this pyridinoline compound. They also examined *Ascaris* cuticle collagen for the presence of pyridinoline and found no appreciable amounts, but specimen age was not reported. Comparison of spectra from particulate material and $NaDodSO_4$-cleaned cuticles in the present study demonstrates that trypthophan fluorescence, when present, can obscure other emission peaks that excite around 300 nm. When emission intensities were normalized to Lowry protein, the pyridinoline-like fluorescence increased 13-fold in older worm samples while the protein tyrosine fluorescence increased nearly 16-fold. However, if ratios of intensities of the pyridinoline-like peaks relative to the protein tyrosine peaks are compared between 4-day and 12-day groups, significant age differences are eliminated. This suggests concomitant increases between the two peaks and may reflect some as yet unexplained interdependence. Monnier & Cerami (1981) review an increased tyrosine content in cataractous lens protein and postulate its origin from modified lysine residues. Hydroxypyridinium (pyridinoline) cross-links have also been shown by radiochemical studies on rabbit cartilage collagen to be derived from lysine residues (Eyre, 1980). The recent discovery of a new cross-linking amino acid, "isotrityrosine", from *Ascaris* cuticle collagen may ultimately be implicated in this phenomenon should it be found to increase with age (Fujimoto et al., 1981).

The remaining fluorescence from $NaDodSO_4$ cuticle preparations, areas S_1, S_2, and X on averaged spectra, may represent Schiff base products of varied composition. In the sequence of events of collagen cross-linking, conjugate Schiff base compounds (aldimines) form initial borohydride-reducible cross-links. These are thought to lead to permanent, nonreducible cross-links possibly represented by pyridinoline as previously discussed (Eyre, 1980).

Thus, the utility of the *C. elegans* model for aging studies has been extended to include collagenous connective tissue proteins. Since Edgar and colleagues have embarked on a detailed study of the *C. elegans* cuticle (Cox et al., 1981a,b), this may prove to be a very good model for studying aging and other alterations of such proteins.

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