Endogenous Skin Fluorescence Includes Bands that may Serve as Quantitative Markers of Aging and Photoaging

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Aging and photoaging cause distinct changes in skin cells and extracellular matrix. Changes in hairless mouse skin as a function of age and chronic UVB exposure were investigated by fluorescence excitation spectroscopy. Fluorescence excitation spectra were measured *in vivo*, on heat-separated epidermis and dermis, and on extracts of mouse skin to characterize the absorption spectra of the emitting chromophores. Fluorescence excitation spectra obtained *in vivo* on 6 wk old mouse skin had maxima at 295, 340, and 360 nm; the 295 nm band was the dominant band. Using heat separated tissue, the 295 nm band predominantly originated in the epidermis and the bands at 340 and 360 nm originated in the dermis. The 295 nm band was assigned to tryptophan fluorescence, the 340 nm band to pepsin digestable collagen cross-links fluorescence and the 360 nm band to collagenase digestable collagen cross-links fluorescence. Fluorescence excitation maxima remained unchanged in chronologically aged mice (34–38 wk old), whereas the 295 nm band decreased in intensity with age and the 340 nm band increased in intensity with age. In contrast, fluorescence excitation spectra of chronically UVB exposed mice showed a large increase in the 295 nm band compared with age-matched controls and the bands at 340 and 350 nm were no longer distinct. Two new bands appeared in the chronically exposed mice at 270 nm and at 305 nm. These reproducible changes in skin autofluorescence suggest that aging causes predictable alterations in both epidermal and dermal fluorescence, whereas chronic UV exposure induces the appearance of new fluorphores. *Key words: spectroscopy/UVB. J Invest Dermatol 111:776–780, 1998*

Characterization of skin fluorescence in terms of the native fluorophores (Sauermann and Hoppe, 1992; Utz *et al*, 1993) has been proposed as a way of differentiating between normal and diseased skin (Cordeiro *et al*, 1994), and between intrinsically aged and photoaged skin (Leffell *et al*, 1988). Endogenous substances in tissue that produce fluorescence upon excitation (fluorophores) include NADH, collagen, elastin, aromatic aminoacids (tryptophan and tyrosine), porphyrins, and FAD (Sauermann *et al*, 1992). Fluorescence spectra of these substances have been characterized *in vitro* (Schomacker *et al*, 1992). With knowledge of the *in vitro* fluorescence emission spectra of these compounds, similar emissions have been investigated *in vivo* (Utz *et al*, 1993; Zeng *et al*, 1995). Fluorescence photography has been used to investigate pigment distribution in photodamaged skin (Kollias *et al*, 1997) and the porphyrin and horn fluorescence of acne involved skin (Lucchina *et al*, 1996). Determination of the fluorescence properties of a fluorophore include measurement of both the excitation and the emission spectra. From these spectra a pair of wavelengths may be determined that correspond to the wavelength of maximum emission intensity when excited at the corresponding maximum of the excitation spectrum. The maxima of these spectra constitutes an "excitation–emission" pair characteristic of that fluorophore. Standards are used to obtain the absolute fluorescence intensity. In tissue there exist multiple spectrally overlapping fluorophores. Identification of a fluorescing molecular species can be better accomplished once the excitation–emission pair for a fluorophore has been determined.

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Laser-induced fluorescence has been used to investigate skin fluorescence *in vivo* (Leffell *et al*, 1988; Zeng *et al*, 1995). Lasers are monochromatic and have high power, eliminating the need for an excitation monochromator and resulting in the delivery of high light intensity. Although this results in significantly easier detection, excitation with a single laser excitation source leads to complicated emission spectra that are a challenge to analyze in terms of the contributions of individual skin fluorophores. The limited number of excitation wavelengths used in laser-induced fluorescence studies together with modifications of the emission spectra by tissue optics (Anderson, 1989) and other factors (Lakowicz, 1983), often make it difficult to separate and identify the fluorophores in a complex spectrum.

Biologic environments may also significantly shift the fluorescence maxima and seriously affect the efficiency with which a molecule fluoresces (Lakowicz, 1983). Fluorescence intensity, which is used to estimate the concentration and location of a fluorophore, is often microenvironment dependent. Furthermore, absorption of *in vivo* fluorescence by hemoglobin and melanin have often been noted (Leffell *et al*, 1988; Anderson, 1989; Lohmann *et al*, 1991). The presence of absorbing chromophores alter the fluorescence emission and excitation spectra, making the characterization and eventual quantitation of fluorophores difficult.

Fluorescence excitation spectra permit the identification of excitation bands associated with specific emission bands. One advantage of fluorescence excitation spectra compared with fluorescence emission spectra is that the former are similar to absorption spectra, which aids in the separation and identification of the individual fluorophores in a complex spectrum. The instrument used in this study purposely avoided the major constraint imposed by laser sources, namely the limited number of excitation wavelengths, by using a continuum light source

(Xe-arc) in combination with an excitation monochromator. The system chosen was based on fiber optic bundles, which allow the study of living systems. The goals of this investigation were to investigate the fluorescence excitation spectra from hairless mouse skin *in vivo* and to identify the spectral changes that take place with aging and photoaging.

MATERIALS AND METHODS

Chemicals Elastase and collagenase were obtained from Elastin Products (Owensville, MO) and pepsin from Sigma (St. Louis, MO), and each was used as received. Monobasic and dibasic potassium phosphate, acetic acid, and sodium hydroxide were obtained from Sigma (St. Louis, MO). All solutions were freshly prepared prior to use.

Animal model The animal model studied was the albino hairless mouse, obtained from Charles River Laboratories (Wilmington, MA).

Fluorescence excitation measurements Fluorescence spectra were obtained with a SPEX Fluorolog model 212 spectrofluorimeter (SPEX Industries, Edison NJ) consisting of a 450 W Xenon arc lamp, double excitation and emission monochromators, and a photomultiplier detector (R928P, Hamamatsu, Hamamatsu City, Japan) connected to a single photon counting system. The instrument bandwidth was 3.8 nm for all measurements. The output of the excitation monochromator was focused onto one leg of a bifurcated quartz fiber optic bundle that brought the excitation radiation to the skin (6 mm diameter bundle, 0.1 mm diameter individual fibers). The joined end of the fiber bundle had randomly mixed illuminating and receiving individual fibers. This end was placed in contact with the skin during the measurement. The fibers that collect radiation from the skin were optically coupled into the emission monochromator. The detector output was displayed and stored on a computer that controlled the operation of the spectrophotometer.

Special care was taken when placing the probe in contact with the skin to minimize the attenuation of the fluorescence emission by hemoglobin. Sufficient pressure was applied to the skin site by the probe to minimize perfusion, and to ensure excellent contact between the measurement device and the skin. This was accomplished by placing the probe along the back of the animal and then raising the skin over the end of the fiber optic bundle probe. A set of eight fluorescence excitation spectra were collected by positioning the emission monochromator from 340 to 480 nm in increments of 20 nm and scanning the excitation monochromator from 260 nm to within 20 nm of the emission monochromator. This series of excitation spectra then permitted us to identify the major fluorescence excitation–emission pairs present in skin.

Experimental groups

Method validation (i) A series of measurements were carried out to check the reproducibility of the fluorescence excitation spectra obtained *in vivo* at various time points during the same day and also on three different days spanning a 7 d period; (ii) a second series of fluorescence excitation spectra were recorded from the upper, middle, and lower back of three different animals; (iii) inter-animal variation for animals of the same age was determined for five animals at one time point and at approximately the same anatomical location on the back of each animal.

Fluorescence excitation spectra were obtained from the skin of three young hairless mice that were then sacrificed by cervical dislocation. Measurements were repeated within 5 min after death. The skin was removed and fluorescence excitation spectra were obtained from intact skin as well as heat separated epidermis and dermis to determine the location of the fluorophores. Heat separation was performed by placing the skin in 60°C water for 30 s and then gently pulling the epidermis from the dermis (Blank *et al*, 1984). The spectra from the heat separated skin were similar to those obtained from skin *in vivo*.

Aging Fluorescence excitation spectra were obtained from four different age groups of mice; young mice (4–6 wk), middle aged mice (≈16–20 wk), old mice (34–38 wk), and very old mice (>45 wk). The fluorescence measurements were repeated on two further groups of five animals, one consisting of young animals (4–6 wk) and one of middle aged animals (16–20 wk), to confirm the previous results.

Photoaging A group of 15 animals were divided into control (five animals) and experimental (10 animals) groups. The experimental group of animals was exposed three times a week for 20 wk to UVB radiation using a bank of 12 equally spaced fluorescent lamps (UVB-HO-90°, Elder Pharmaceuticals, Bryan, OH). The total dose delivered was 6.8 J per cm^2. The animals were placed at a distance of ≈40 cm from the lamps to ensure a uniform irradiance of 0.5 mW per cm^2 during exposure. The irradiance was measured using a IL1700 radiometer equipped with a SED 240 UVB detector (International Light, Newburyport, MA) prior to each irradiation. The spectrum of the lamps was measured periodically with a spectroradiometer (model 742, Optronix Laboratories, Orlando, FL). The spectral output consisted of 3.5% between 260 and 290 nm, 76.8% between 290 and 320 nm, and 19.7% between 320 and 400 nm. Mice were irradiated every other day (Monday, Wednesday, Friday) (Kochevar *et al*, 1993).

Fluorescence excitation spectra were obtained from five of the experimental animals prior to the treatment and twice during the treatment. Nine chronically irradiated animals and five age-matched control animals were measured at the end of the treatment. Varied degrees of inflammation were observed on the backs of the experimental animals at the time of the last measurement.

Skin extraction procedure to evaluate the fluorescence spectra associated with various components The general procedure of Miller and Rhodes (1982) was followed. A total of 4 g of dorsal skin from three mice (6 wk old) was homogenized with ice cold 0.1N NaOH, dialyzed at 4°C overnight, then centrifuged. The supernantant was removed and stored at –20°C. The pellet was extracted a second time with 0.1N NaOH. These extractions removed the cellular and soluble components from the homogenized skin. Fluorescence excitation spectra were measured on the combined supernatants and the pellet was suspended in distilled water.

The pellet was washed twice in distilled water, then extracted twice overnight with 0.15 M phosphate buffer. These extractions removed neutral collagen (not cross-linked) from the pellet. Fluorescence measurements were obtained from the supernatant and the pellet was suspended in distilled water. After washing twice in distilled water, the pellet was treated with 0.5 M acetic acid overnight, which removed acidic components of collagen from the pellet. Following centrifugation, the fluorescence from the supernatant and the resuspended pellet was measured. After washing, the pellet was treated overnight with 0.5% pepsin at 37°C in 0.5 M acetic acid, which degraded and removed the insoluble cross-linked collagen. Following centrifugation, the fluorescence of the supernatant and pellet was measured. The pellet residue was then treated with collagenase at 37°C in 50 mM phosphate buffer (pH 7.5) overnight, which removed the remaining collagen, then centrifuged; fluorescence was measured on the supernatant and suspended pellet.

RESULTS

Cutaneous fluorescence spectra are reproducible and are composed of three major bands All fluorescence excitation spectra shown have been corrected for the source and system spectral response. **Figure 1** shows the average fluorescence excitation spectrum obtained from five young mice. Three bands are observed in this spectrum: at 295 nm, at 340 nm, and at 360 nm. The 295 nm band has the highest relative intensity, followed by the 360 nm band and then by the 340 nm band. The spectra obtained from the five different young animals were all nearly identical. The spectral location and also the ordering of the relative intensity of the excitation bands within any group of animals always remained constant. Inter-animal variation, site to site variation, or variations at measurement time points within a period of 7 d never exceeded 10% of the average intensity for any one band.

Fluorescence excitation spectra of epidermis and dermis occur over distinct spectral regions The epidermis and dermis were investigated separately *ex vivo* from young mice to identify the location by skin layer of the major fluorophores. The fluorescence excitation spectra of the epidermis, **Fig 2(*a*)**, show a band with a very clear maximum at 292 nm, which is sharper at its peak than the *in vivo* measurements; however, the band extends further to the shorter wavelengths yielding a somewhat broader 295 nm band than obtained from young mice *in vivo*. Bands at 340 nm and 360–370 nm are essentially absent from these spectra. The fluorescence excitation spectra from the dermis, **Fig 2(*b*)**, show distinct bands with substantial maxima at 340 nm and 370 nm as well as a sharp maximum at 292 nm with a substantially greater shoulder to shorter wavelengths than the epidermis.

The 335–340 nm fluorescence excitation maximum belongs to pepsin digestible collagen cross-links Fluorescence excitation spectra obtained from extractions indicate that the 295 nm fluorophore was easily extracted from the suspended solids into the supernatant. The 335–340 nm band is attributed to collagen cross-links because they remained present in the first few extractions and completely

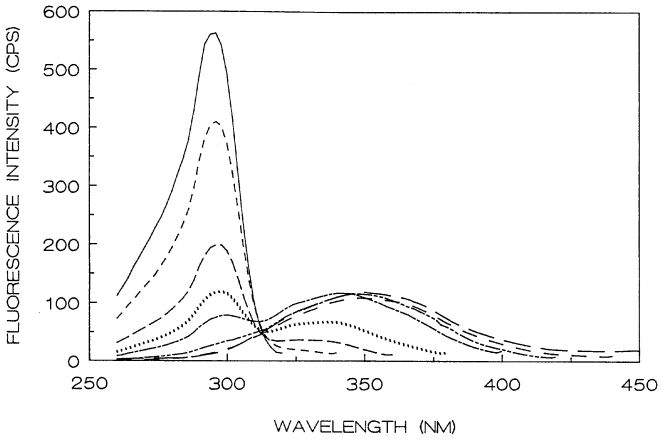


Figure 1. Serial fluorescence excitation spectra from skin show three major bands. Serial fluorescence excitation spectra consist of eight spectra. The first spectrum was obtained by setting the emission monochromator to 340 nm and scanning the excitation monochromator from 260 nm to 20 nm short of the emission wavelength, i.e., 320 nm. In the second spectrum the emission monochromator was advanced by 20 nm to 360 nm and the excitation monochromator was scanned from 260 nm to 20 nm short of the emission wavelength, i.e., 340 nm. All the other spectra were obtained following this pattern, sequentially increasing the emission wavelength by 20 nm and the range of the excitation scan by 20 nm. The spectra were obtained from 6 wk old hairless mice with the fiber bundle in direct contact with the skin. Similar spectra were obtained from all animals of the same age within 10%. ——, Emission at 340 nm; – – – –, emission at 360 nm; —— —— ——, emission at 380 nm; · · · · ·, emission at 400 nm; —— – —— –, emission at 420 nm; —— – – —— – –, emission at 440 nm; — — — emission at 460 nm; —— —— ——, emission at 480 nm. Fluorescence intensity was measured in counts per second (CPS).

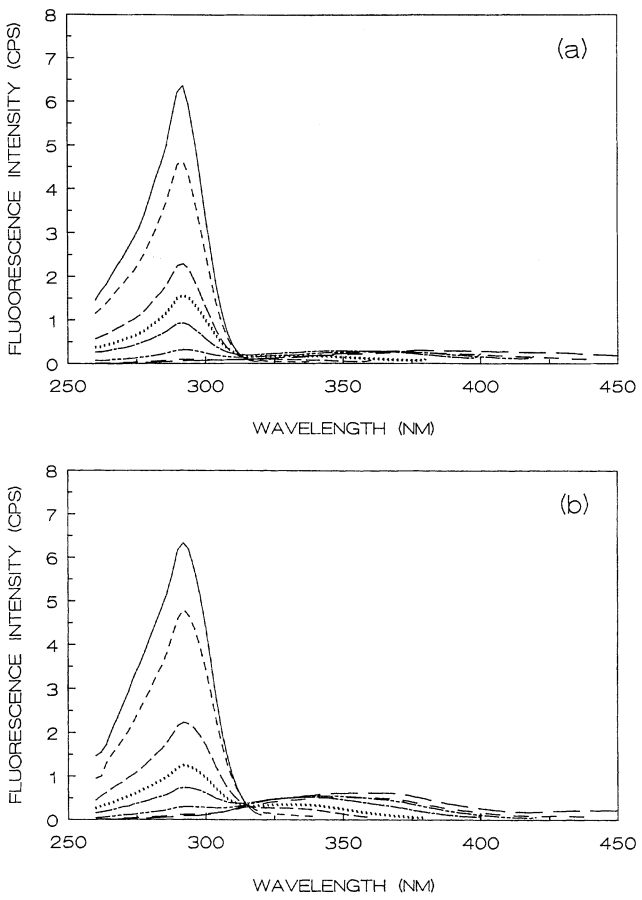


Figure 2. The fluorescence of epidermal origin occurs in a different wavelength band than the fluorescence of dermal origin. Serial fluorescence excitation spectra from heat-separated (*a*) epidermis and (*b*) dermis. Note that the spectra are somewhat different from the *in vivo* spectra. The 295 nm tryptophan band is due to epidermal components and the 320–420 nm bands are due to dermal elements such as collagen cross-links and elastin. The 295 nm band in the dermal spectra is probably attenuated entirely *in vivo* by the proteins in the epidermis. The serial spectra from intact skin cannot be reconstructed by a simple addition of the component spectra. ——, Emission at 340 nm; – – – –, emission at 360 nm; —— —— ——, emission at 380 nm; · · · · ·, emission at 400 nm; —— – —— –, emission at 420 nm; —— – – —— – –, emission at 440 nm; — — — emission at 460 nm; —— —— ——, emission at 480 nm. Fluorescence intensity was measured in units of 1000 counts per second (CPS).

disappeared after the pepsin digestion, **Fig 3**. The excitation maxima at 355 and longer wavelengths remained unaffected by the extractions.

The 295 nm band decreases and the 335–340 nm band increases with aging The average fluorescence excitation spectra from five mice, 18 wk old, are shown in **Fig 4**. As the animals aged, the relative intensity of the 295 nm band decreased and the relative intensity of the 340 nm band increased. Further, the maximum of the 360 nm band in the young mice spectra shifted slightly towards 370 nm as the animals aged. The fluorescence excitation spectra obtained from a very old mouse (age >52 wk) further accentuate the above differences. The relative intensity of the 295 nm band is further decreased, the 340 nm band is further increased substantially, and the 360–370 nm band is increased slightly. These changes are apparent by comparing **Figs 1**, **4**, and **5**(***b***). It may be noted that as these animals naturally age the intensity of the 295 nm band decreases, the intensity of the 340 nm band increases, and the intensity of the 360 nm band remains essentially unchanged.

Chronic UVB exposure induces additional fluorescence excitation bands The average fluorescence excitation spectra from nine photoaged mice are shown in **Fig 5**(***a***). The average fluorescence excitation spectra from five age-matched control mice are shown in **Fig 5**(***b***), these mice were 30–32 wk old. Compared with the control mice, the relative intensity of the 295 nm band of the photodamaged animals is 800% greater. Additional bands at ≈270 nm and at 305 nm are seen in the spectra from the photoaged mice that are absent in the spectra from the control mice. In the photodamaged mice, there is also a broad band centered about 355 nm, whereas for the control mice there is the distinct appearance of both the 340 nm and the 360 nm bands.

DISCUSSION

This study shows distinct qualitative and quantitative changes in cutaneous fluorescence due to aging and chronic UVB exposure (photoaging). The dominant epidermal excitation band at 295 nm *in vivo* is attributed to tryptophan fluorescence (Sauermann *et al*, 1992). Spectra obtained *ex vivo* from samples of epidermis, dermis, and intact skin showed a blue shift of this band from 295 to 292 nm. Although the relative abundance of tryptophan in the skin is small, it has a high fluorescence yield in the skin. There are no other amino acids with significant fluorescence in this spectral range. The fluorescence signals from tryptophan appear to be strong from both the stratum corneum and the viable epidermis. The stratum corneum fluorescence is not detected in the fiber arrangement used in this study. The fluorescence signals that originate in the stratum corneum do not find their way into the receiving fibers because the excitation fibers are in contact with the stratum corneum.

The band at 335–340 nm is attributed to the fluorescence of collagen cross-links (Deyl *et al*, 1970; Monnier *et al*, 1984; Odetti *et al*, 1992), which we have shown are pepsin-digestable. Our results show that the band at 360 nm is associated with collagenase digestible collagen cross-links. This band undoubtedly has contributions from elastin and/or NADH fluorescence as well, which overlap spectrally (Cordeiro *et al*, 1994). The NADH contribution to the 360 nm band, however, is probably small as this band did not change significantly when hypoxia was induced by pressure occlusion or when the animal was sacrificed; this fluorescence is therefore likely to be mainly from collagenase-

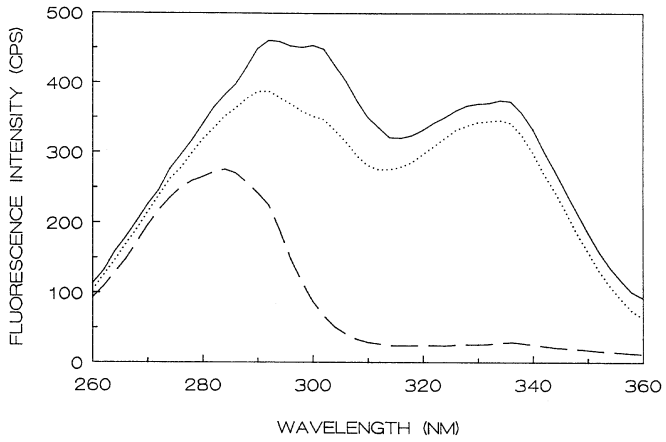


Figure 3. Pepsin-digestible collagen cross-links are the source of one of the principal fluorescence maxima from skin. The fluorescence spectra of skin extracts change only following pepsin digestion. Fluorescence excitation spectra obtained from suspended solids following base and acid extraction show the same excitation maximum at 335 nm when emitting at 380 nm. Following pepsin extraction the fluorescence of the suspended solids at 335 nm becomes very small, extraction with collagenase did not further alter this fluorescence band. All curves are excitation spectra with emission at 380 nm: ———, fluorescence of the solids following base extraction; · · · · · , fluorescence of the solids following acid extraction; — — —, fluorescence of the solids following pepsin digestion. Fluorescence intensity was measured in counts per second (CPS).

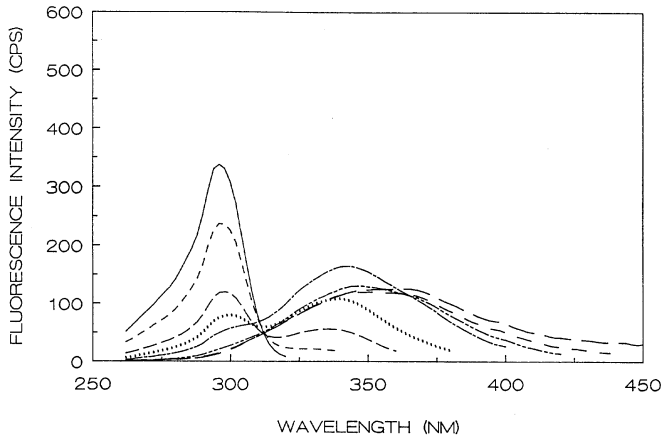
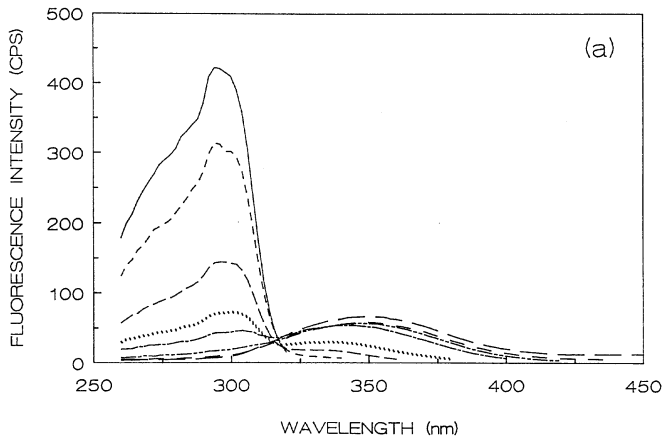


Figure 4. The magnitude of the tryptophan fluorescence and the pepsin-digestible collagen cross-link fluorescence maxima change consistently with aging. Serial excitation spectra obtained as outlined in **Fig 1** from the skin of aged (18 wk) hairless mice. The 295 nm tryptophan band is decreased, compared with **Fig 1**, whereas the 335 nm band becomes prominent with age and the 350–370 nm band remains essentially the same. ——, Emission at 340 nm; – – – –, emission at 360 nm; —— —— ——, emission at 380 nm; · · · · · , emission at 400 nm; —— – —— –, emission at 420 nm; —— – – —— – –, emission at 440 nm; — — — emission at 460 nm; —— —— ——, emission at 480 nm. Fluorescence intensity was measured in counts per second (CPS).

digestible collagen cross-links and elastin. These conclusions are further substantiated by the spectra from heat-separated epidermis and dermis.

Tryptophan fluorescence (295 nm) was observed from both the epidermis and the dermis when the two were separated. A substantial collagen cross-link and elastin fluorescence (340 and 360 nm) was observed from the dermis, whereas negligible fluorescence from these fluorophores was observed from the epidermis. The weak epidermal fluorescence of these fluorophores is due either to keratins or to dermal elements from imperfect separation of the skin. It is important to note that the sum of the spectra obtained from the epidermis and from the dermis *ex vivo* do not yield the spectrum of intact skin. This is mainly due to skin optics. At wavelengths shorter than 310 nm, more than 90% of the incident radiation is attenuated in the stratum corneum and the viable epidermis. Thus the epidermis is optically thick at



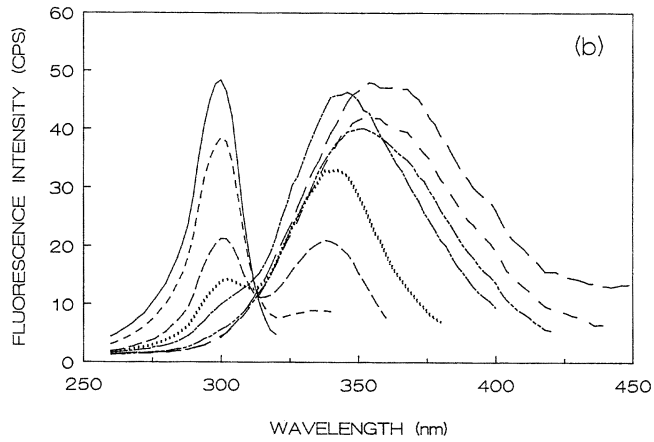


Figure 5. The fluorescence spectra of photoaged skin show a large increase in tryptophan and a disappearance of the collagen-digestible collagen cross-links as well as the induction of new fluorophores. Serial excitation fluorescence spectra from (*a*) photoaged skin (n = 9) and (*b*) age-matched control skin (n = 5) of hairless mice. The spectra obtained from photoaged skin show additional bands at 270 and at 305 nm, as well as a 295 nm band that is approximately 10 times larger than in the control animals. The spectra obtained from the control animals show a prominent 335 nm band along with a significantly reduced in size 295 nm band. Inflammation was evident in the skin of the photoaged animals. ——, Emission at 340 nm; – – – –, emission at 360 nm; — — —, emission at 380 nm; · · · · · , emission at 400 nm; —— – —— –, emission at 420 nm; —— – – —— – –, emission at 440 nm; — — — emission at 460 nm; —— —— ——, emission at 480 nm. Fluorescence intensity was measured in counts per second (CPS).

295 nm, which makes it difficult to obtain measurable fluorescence signals from the dermis *in vivo*. The number of photons that do traverse the epidermis, however small, is sufficient to produce biologic activity as erythema. The shift in the fluorescence spectra from the heat-separated epidermis and dermis is probably due to changes in the pH of the tissue.

Chronologic aging produces changes in the fluorescence intensity of the tryptophan and the pepsin-digestible collagen cross-links Considerable care was taken in these experiments to minimize hemoglobin absorption of autofluorescence by obtaining duplicate spectra using diascopy or increasing the pressure of the probe of the skin, therefore changes in the spectra cannot be due to changes in hemoglobin concentration in the investigated sites. As the mice aged, the typtophan fluorescence decreased and the pepsin digestible collagen cross-links fluorescence increased, accompanied by a slight increase in the collagenase digestible/elastin fluorescence. Mice of ≈70 wk of age showed substantial pepsin digestible collagen cross-links fluorescence and minimal tryptophan fluorescence, the reverse of the fluorescence intensities observed for young mice. These results show that as the mice age, the epidermal proliferation measured by the 295 nm fluorescence decreases, whereas collagen cross-linking

measured by the 335–340 nm fluorescence increases. The relative fluorescence intensities of these fluorophores were indicative of the age of the mouse.

Photoaging induces an increase in tryptophan fluorescence and a decrease in pepsin-digestible collagen cross-links fluorescence as well as new fluorophores The tryptophan fluorescence of the photodamaged mice was significantly greater (≈800%) than that from the age-matched control mice. Chronic UVB exposure induced higher proliferative activity in the epidermis, resulting in a substantial increase in epidermal thickness (Kochevar *et al*, 1993), approximately three times that of age-matched control animals. The chronically exposed animals also showed a decrease of the pepsin-digestible collagen cross-links fluorescence, whereas little change was observed for the collagenase digestible collagen cross-links fluorescence. In these spectra we also find the appearance of new bands at 270 nm and 305 nm. The fluorophores responsible for these signals have not been identified, it is suspected that the 270 nm signal is due to tryptophan and appears consistently when a substantial degree of inflammation is induced in the skin such as is found in psoriasis or in skin cancer. The 305 nm signal has not been found in reactions due to acute insults to the skin. It appears to be due to the accumulation of some substance with chronic exposure or chronic inflammation. We can thus conclude that the natural aging process produces changes in the apparent concentration of the fluorophores, whereas photodamage produces changes both in the apparent concentration of the native fluorophores as well as in the appearance of new fluorophores.

Fluorescence excitation spectroscopy has proved very useful in identifying in skin spectra reproducible fluorescence excitation–emission pairs, enabling their use to characterize and quantitate aging. We intend in future studies to use these excitation–emission pairs to further investigate and correlate the fluorescence with the relative abundances of tryptophan, pepsin-digestible collagen cross-links, and collagenase-digestible collagen cross-links, as well as elastin and NADH in hairless mouse skin. In this study a methodology was developed for the use of fluorescence excitation spectroscopy to characterize the intrinsic fluorescence of the skin of the hairless mouse. Similar techniques are now being used to obtain fluorescence excitation spectra from human skin *in vivo* and *ex vivo*. Fluorescence excitation spectroscopy thus gives us the possibility of following markers in the skin such as tryptophan, and collagen cross-links. It also gives us the possibility of studying dynamic changes in these parameters in both healthy and diseased tissue, and to investigate modifications in the concentrations of these chromophores as well as the induction of others following insults to the skin.

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