Continuation of G-33—A12

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NEW WITH THIS CHANGE
Project No. G-33-A12 (Q5250-2A0) School/Dept. Chemistry
Includes Subproject No.(s) N/A
Project Director(s) N. T. Yu ETR/GIT
Sponsor DHHS/PHS/NIH/National Eye Institute
Title Comparative Raman Studies of Human and Animal Lenses

Effective Completion Date: 4/30/88 (Performance) 7/31/88 (Reports)

Grant/Contract Closeout Actions Remaining:

[ ] None
[ ] Final Invoice or Copy of Last Invoice Serving as Final
[ ] Release and Assignment
[ ] Final Report of Inventions and/or Subcontract:
  Patent and Subcontract Questionnaire sent to Project Director [ ]
[ ] Govt. Property Inventory & Related Certificate
[ ] Classified Material Certificate
[ ] Other

Continues Project No. G-33-A12 Continued by Project No. G-33A13

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Thus, this reflective procedure will yield data comparable to our optical dissection used on intact living lenses (Asken, Yu and Kuck, 1979); yet is clearly applicable to lens tissue which contains too much opacity to be analyzed in the transmission mode.

C. Progress Report/Preliminary Studies
   (i) Period: May 1, 1983-May 31, 1987
   (ii) Professional personnel who have worked on the project: Yu, Nai-Teng, Professor (Principal Investigator) 05/01/83-present, 10% academic year plus 30% summer months.
   Bando, M., Senior Res. Associate, 10/01/81-09/30/83, 100%
   Barron, B., Graduate Res. Assistant, 05/01/83-present, 100%
   DeNagel, D. C., Graduate Res. Assistant, 05/01/83-present, 100%
   Cai, M.-Z., Research Associate, 10/01/86-present, 100%
   Kuck, John F. R., Jr., Research Collaborator (Emory Univ.) 05/01/83-present.
   (iii) Previous applications specific aims: We have proposed to achieve the following specific aims: 1) To further improve our in situ Raman technique for the measurement of -SH level and rate of red fluorophor production (for both animals and humans); 2) To develop an automated Raman/fluorescence surface scanning system (instrumentation and procedures) for routine analysis of precise distributions of lens constituents and various fluorophors; 3) To investigate the effects of repetitive near UV exposure on the -SH level and fluorophor production in the lens of rabbit eye and to test the hypothesis that in a non-rodent system the -SH level falls at an accelerated rate long before the appearance of visible opacity; 4) To examine whether 3-OH kynurenine reacts photochemically with α, β, and γ-crystallins and whether new fluorophors are formed by the reaction; 5) To determine the difference among the three crystallins in regard to the photoreactivity with 3-OH kynurenine; 6) To investigate the photo-reactivity and formation of new fluorophors from 3-OH kynurenine which has been incorporated into the rat lens; 7) To compare the fluorescence properties of those fluorophors formed by photoreaction with 3-OH kynurenine and the red fluorophors detected in older and brunescent human lens by laser Raman instrumentation; 8) To explore the nature of the electronic state responsible for the emission of red fluorescence in the 3-OH kynurenine photoprodut with crystallins; 9) To study variations of total sulfhydryl along visual axis for normal human lenses between 68 and 100 years of age; (10) to obtain Raman evidence for the formation of disulfide bonds and methionine sulfoxide in human cataractous lenses).

   We have achieved most of the specific aims and generated a total of 12 papers (7 in print, 5 in press or in revising stage) and another six manuscripts in preparation. The aim #1 has been expanded and developed into a new project "Clinical Monitor of Diabetic Lenses by Fluorescence/Raman" (EY 07006-01) which has been funded, starting 04/01/87. It is now possible to bypass the stage of animal (rabbit) testing and apply directly the in vivo (or in situ) Fluorescence/Raman technique for clinical evaluation (in collaboration with Dr. Sven-Erik Burssell of Joslin Diabetes Center). Since the submission of our previous application, we discovered that the age-related changes in SH and SS in guinea pig (unlike rat and mouse) are very similar to those in rabbit. The aim #3 has been achieved in our noninvvasive Raman studies of -SH, Trp, SS, and fluorophors as affected by long-term and short-term near UV-
irradiation (see papers #7 and 8). We are very proud of our achievement in aim #2 in which we have delivered we promised in developing an automated Raman/fluorescence surface scanning spectrometer for image analysis of precise distributions of lens fluorophors and constituents. The total cost for assembling this unique system is over $90,000, which far exceeds the approved equipment budget ($13,033) excluding laser plasma tube replacements. We achieved the aim #2 by rebudgeting and by using the equipment funds available from other sources. The design and performance of this automated laser scanning microbeam fluorescence/Raman system are reported in ARVO (1987), Microbeam Analysis Meeting (07/12-07/16/87; Hawaii) and 2nd Beijing Conference and Exhibition on Instrumental Analysis (10/20-10/23/87; Beijing, China). The utility of the technique in obtaining evidence for metabolic production of a green fluorophor in human lens has been demonstrated in paper #10. The specific aims #4-7 have been achieved and reported in papers #2 and #3. The specific aims #8 and 9 are partially achieved and experimental data are still under analysis. Finally, we have succeeded in detecting the formation of disulfide bonds in Tibet cataractous lenses and normal lenses older than 75 years old. However, we have not yet detected the formation of methionine sulfoxide in human cataractous lenses.

(iv) Summary of progress and significant findings: (a) Development of automated laser scanning microprobe spectrometer with multichannel detection (papers #10 and 11)

This is the most significant accomplishment during this grant period. Now we have a unique instrumentation (Fig. 1) and methodology for performing fluorescence/Raman image analysis of both human and animal lenses. This new

![Fig. 1](image)

FOR COLOR PICTURES, SEE APPENDIX.
system is capable of (1) multichannel detection of "position-defined" fluorescence/Raman spectra from gridded points (1-8 μm), (2) scanning both micro (10 μm x 10 μm) and macro (2.5 cm x 2.5 cm) samples, (3) automated simultaneous acquisition of intensity data of up to six spectral lines (either peak or integrated intensity) from each point, (4) excellent stray-light rejection to allow detection of weak Raman lines from solid samples, (5) normalization of fluorescence intensity with Raman signals (fluorescence/Raman intensity ratio) and (6) presentation of the X-Y data set in three-dimensional perspective, three-dimensional perspective with cursor-sectioning, six-color map representing regions of different intensity intervals, and topographic contour map with lines intersecting the intensity data with constant height intensity planes. It has been employed to make fluorescence intensity measurements from a total of 1,200 points in a human lens section. The precise geometric distribution profile of a green fluorophor (emission maximum at 520 nm with excitation between 400-450 nm) has been constructed for both young (5-month-old) and old (73-year-old) lenses (see Fig. 2). This age-dependent fluorophor is present in the infant lens, in addition to the major lens fluorophor (3-OH L-kynurenine-0-8-glucoside) which is known to be metabolically produced (van Heyningen, 1973) and characterized by an emission maximum at 440 nm when excited at 350 nm (Bando, Nakajima and Sato, 1981). We have demonstrated the striking differences in the distribution profile between the young and old lenses. In the young lens, the fluorescence intensity is low (max. 1,489 counts) and the profile exhibits a cone-shape with its maximum near the lens center. In contrast, the old lens exhibits strong fluorescence (max. 24,985 counts) and the profile shows a distinct saddle-shape, displaying a central plateau with a depressed inner region and two unequal maxima near the equatorial cortical regions. A strip of minor local maximum lying along the equatorial axis within the depressed inner region has also been detected. The geometric characteristic of the distribution profile in the old lens does not indicate that the production of this green fluorophor is associated with photocatalyzed reactions by ambient light; it is purely a metabolic product. Moreover, along the lens optical axis the concentration is lower on the anterior side than on the posterior side. This apparent bleaching effect may be caused by the ambient light or metabolically by diffusion from the cortex of substances reacting with the fluorophor.
We discovered that guinea pig lens is very similar to rabbit and human lenses with respect to age-dependent changes in nuclear -SH and S-S. We have obtained direct evidence from Raman spectroscopy that unexpected and remarkable differences with respect to these groups exist between the mouse lens and the lenses of guinea pig and man (see Fig. 3). The mouse lens nucleus exhibits a precipitous fall in the -SH concentration on aging from 1 to 6 months; concomitantly, there is a rise in S-S of comparable magnitude, indicating a direct conversion. The guinea pig lens, however, is quite different with respect to the age-dependent change in nuclear S-S: there is none between 6 months and 5 years. In the human lens S-S behaves exactly as in the guinea pig lens: the level is low and does not change with age between 9 and 65 years. With respect to nuclear SH, these two latter species of lenses show some decrease with age but nothing like the approach to zero found in the aging mouse lens nucleus. The relatively high level of S-S in the old but clear mouse lens does not support the idea that protein aggregation involving formation of intermolecular S-S bonds is necessarily an important cause of nuclear cataract. The small but significant age-related depression of -SH in guinea pig lens nuclei without any accumulation of S-S may be explained as a result of glutathione oxidation and subsequent extrusion of glutathione disulfide by the lens. We proposed that the oxidation of glutathione proceeds by reaction with protein disulfide groups to yield protein sulfhydryl and a mixed disulfide of glutathione and protein; the mixed disulfide is capable of being reduced by glutathione reductase and NADPH, yielding the original PSH and GSSG, which is extruded from the lens. From this study, we found that guinea pig lens is a good animal model for understanding the behavior of nuclear SH and S-S in human lens aging and under the influence of ambient near UV radiation.

Fig. 3

VA disulfide profiles for mouse (a), guinea pig (b), and human (c) lenses at several ages.

VA sulfhydryl profiles for mouse (a), guinea pig (b), and human (c) lenses at several ages.
(c) Effects of near UV on tryptophan, -SH, S-S and fluorescence (papers #7 and 8)

The laser Raman/fluorescence optical dissection technique was used to measure the Trp, -SH, S-S and fluorescence of distinct points along the visual axis of the guinea pig lens after aging and long-wave UV exposure (9 months duration, in vivo). We have obtained important results: (1) the loss of sulfhydryl can be accelerated in the guinea pig lens by in vivo UV exposure for 9 months; (2) there is a subsequent uniform increase in the disulfide content across the visual axis after UV exposure suggesting a direct sulfhydryl to disulfide conversion in the guinea pig lens; (3) there is no detectable Trp photolysis in the inact guinea pig lens by longwave UV light in vivo (long-term UV light; 9 months, 353-nm peak) or in vitro (short-term UV light; 3.5 hours, 325-nm line from a He-Cd laser); and (4) the 457.9-nm-excited fluorescence of the lens increases by longwave UV-irradiation. The maximum increase was in the nucleus and the least effect was in the posterior cortex.

(d) Metabolic production of a blue-green fluorophor in the mouse lens (paper #1)

We have shown that a blue-green fluorophor (emission maximum at 496 nm with excitation at 406.7 nm) of the mouse lens occurs at the same concentration in mice reared in the dark from birth as in mice reared under the cool-white fluorescent lighting (12 hrs/day) conditions. Thus, this fluorophor is clearly a metabolic product with no relationship to photochemical effects. It exhibits a steady increase in intensity (hence concentration) with age.

(e) The unique methodology of fluorescence/Raman intensity ratio measurements (paper #3)

We have put quantitation of human lens fluorescence on a rational basis by using the accompanying Raman signal from lens protein as a normalization factor. The fluorescence/Raman intensity ratio measurements provide artifact free measurements of the various long wavelength lens chromophore fluorescences in the excised lens. The importance of this work is that it leads to the development of a clinical instrument capable of making artifact free autofluorescence measurements from the living human lens (in collaboration with Dr. Sven-Erik Bursell of Joslin Diabetes Center).

The fluorescence/Raman ratio may be used to compare lenses of different ages when the exciting wavelength is long enough to give a measureable Raman signal. In younger lenses excited at 457.9 or 514.5 nm, the F/R ratio shows a log increase with age. Older lenses, above 60 years of age, excited at 647.1 nm give a steeply rising sigmoid curve (Fig. 4). For each lens there is a characteristic wavelength that is called the critical wavelength ($\lambda_{\text{critical}}$). At wavelengths longer than $\lambda_{\text{critical}}$ the Raman signal appears in the absence of a broad fluorescence peak; at shorter wavelengths the fluorescence intensity increases enough to overwhelm the Raman signal. For normal lenses, the $\lambda_{\text{critical}}$ is age dependent, giving a curve that is a flattened sigmoid approaching a straight line.
(f) Formation of chromophors and fluorophors from reactions of hydroxykynurenine and lens crystallins (paper #2)

We have investigated the UV-effects on generation of fluorescent chromophors associated with lens crystallins in the presence of 3-hydroxy-L-kynurenine. The protocol proposed in the previous application has been found to work very well. Isolated α-, β-, and γ-crystallins from young rat lenses were incubated in solution for 16 hrs. with 3-hydroxy-L-kynurenine under ultraviolet (366 nm) light. Controls included: incubation without light, without kynurenine, and with 2-mercaptoethanol. These procedures generated several chromophors (with absorption maxima or shoulders at 340, 370 and 470 nm) and fluorophors (with excitation/emission at 407/515, 458/550, 515/555, 647/664 and 647/740 nm). The formation of these pigments was inhibited by 2-mercaptoethanol. The findings have been discussed (in paper #2) in relation to the chromophors and fluorophors found in aged and brunescent human lenses.

(g) We have obtained beautiful Raman spectra of Tibet cataractous lenses using 647 nm-excitation (Fig. 5), in collaboration with Prof. Tian-Sheng Hu of Peking Union Medical College. We have reported, for the first time, the direct evidence for the solar UV-induced lowering of sulfhydryl level in the lens nucleus (see Proc. Int. Soc. Eye Res. Vol. IV, p. 84; Nagoya, Japan 1986).
Preliminary Results

This section presents experimental data from our preliminary studies, which demonstrate the feasibility and interesting aspects of the problems and thus form the important basis for further studies as proposed in this application.

(a) Three-dimensional perspective fluorescence profiles of Tibet and Beijing cataractous lenses (non-equatorial section)

We have investigated the precise distributions of the green fluorophor (emission maximum at 520 nm when excited at 441.6 nm) in the cataractous lens sections. In Fig. 6 we display three perspectives: (a) Tibet (cortical; 67-year old), (b) Tibet (nuclear; 68-year old) and (c) Beijing (nuclear; 68-year old). Striking differences exist between Tibet (cortical) and Tibet (nuclear) and between Tibet (nuclear) and Beijing (nuclear). The most interesting is the comparison between Tibet (either cortical or nuclear cataracts) and Atlanta (normal; 73-year old; see Fig. 2(g)). The interpretations of these differences must await further detailed studies.

(b) Fluorescence profiles on human lens equatorial plane

In spite of the circular nature of the lens equatorial section, we found
that the distributions of metabolic fluorophors are not circularly symmetric. As shown in Fig. 7 the three-dimensional perspectives of the 71-year old lens (Atlanta, normal) (Fig. 7a) and 83-year old lens (Atlanta, normal) (Fig. 7b) exhibit a central depression with uneven cortical fluorescence intensities. It should be interesting to determine if the nasal side has a lower (or higher) intensity. For our future work, we will make efforts to identify if the lens is from right eye (or left eye) and also the mark the nasal side when we remove the lens from the eye.

(c) Comparison of Raman spectra between Tibet and Beijing cataractous lenses

We have detected the disulfide stretching vibration at 503 cm⁻¹ in Tibet and Beijing (cortical) cataractous lenses with relatively clear nucleus. The spectra shown in Fig. 8 were taken from the clear nucleus regions. The 70-year old Tibet lens exhibits a stronger intensity at 503 cm⁻¹ than the 74-year old Beijing lens.
Publications (1983-87) which acknowledge the support of EY01746 grant:


*Reprints/preprints are provided in the Appendix.