GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF RESEARCH ADMINISTRATION

RESEARCH PROJECT INITIATION

Date: March 3, 1975

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Project No: G-33-601 (Continuation of work previously budgeted under G-33-672)

Principal Investigator Dr. Nai-Teng Yu

Sponsor: DHEW/PHS/NIH - National Institute of General Medical Sciences

Agreement Period: From 9/1/74 Until 8/31/78 (End current proj. period)

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RA-3 (6-71)

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SPONSORED PROJECT TERMINATION

Date: September 17, 1976

Project Title: Laser-Excited Raman Spectroscopy of Biopolymers

Project No: G-33-601 (Continued by G-33-G01)

Project Director: Dr. Nai-Teng Yu

Sponsor: DHEW/PHS/NIH National Institute of General Medical Sciences

Effective Termination Date: 8/31/76

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CA–4 (3/76)

Yu, Nai-Teng
585-28-9345

Research Plan for "Comparative Raman Studies of Human and Animal Lenses"

A. Introduction

1. Objectives

The overall objective of the present proposal is to employ the well-developed laser Raman scattering technique for systematic structural studies of intact human and animal lenses (normal and in various pathological states) and their isolated lens constituents, particularly the proteins. The vertebrate species under investigation will tentatively include: Amphibia (frog), Reptiles (snake, turtle), Birds (pigeon, chicken, duck, turkey), Fishs (dogfish, trout, lamprey) and Mammels (java, bovine, rabbit, guinea pig, rat, whale, bat, cat, dog). For comparison, we will also include invertebrate species such as squid, although immunochemical evidence indicates that the proteins of the invertebrate lens are not related to those of vertebrate lenses.

Special attention will be focused on unusual lens proteins from 'birds' as they have a low incidence of cataract. The structure-function relationship of birds δ-crystallin will be fully elucidated.

The role of sugar in the formation of albuminoid during aging process is of considerable interest and will be investigated since I have obtained Raman spectra containing a signal due to protein-bound sugar.

2. Background

The application of laser Raman spectroscopy to naturally occuring proteins as pioneered by Lord and Yu (1970 a,b) has become an area of intense activity (Lord, 1971; Lord and Mendelshon, 1972; Chen, et al. 1973; Chen et al. 1974; Nakanishi et al. 1974; Yu, 1974; Yu et al. 1975; Murphy and Thomas, 1975; Frushour and Koenig, 1974). A preliminary investigation of a bovine lens by this technique was first made by Yu et al. (1974). More detailed studies of both intact lens and its isolated protein fractions have also been published (Yu and East, 1975). These studies were designed to demonstrate the power of non-resonance Raman scattering technique in extracting significant structural information of proteins from an intact biological specimen. We are now ready to expand the scope and ask support from NIH for more systematic studies of human and animal lenses by both non-resonance and resonance Raman methods.

The kinds of structural information obtainable from Raman spectra may be summarized in Table 1.

The ocular lens can be kept in a living state (i.e., metabolically active) in a T-K culture medium (Thoft and Konoshita, 1965) for about 3-4 days. It is an organ which is an exquisite collection of only one type of cells which have no blood vessels or nerves to confuse the picture. The concentration of protein is rather high.

Table 1

Selected Raman Lines Useful for Structural Studies of Ocular Lens

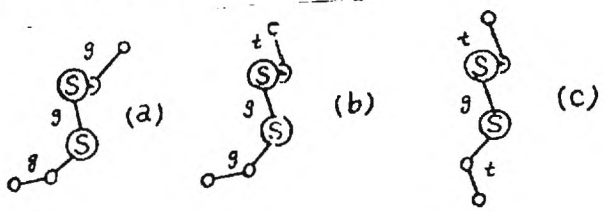
Line (cm^{-1})	Interpretation	References
496	Increase in intensity signifies increase in protein-bound sugar.	unpublished results
510/525/540	ν(S-S); Frequency depends on dihedral angles around two C-S bonds: (a) gauch-gauch-gauch, 510 cm^{-1}, (b) trans-gauch-gauch, 525 cm^{-1} and (c) trans-gauch-trans, 540 cm^{-1}. 	37, 42, 43
622	Phe in-plane vibration; In non-resonance Raman spectra its intensity is not sensitive to micro-environment; a good internal reference for conformational studies of proteins.	56, 64
644	Tyr; Its intensity depends on state of aggregation.	58
630-760	ν(C-S) of disulfide linkage and methionine; The C-S stretching frequencies of X-C-CH_2-S-S depend on the atom X of the trans site with respect to the sulfur atom about the C-C bond; for X=H atom, ν(C-S) at ~660 cm^{-1}; for X=C, ~720 cm^{-1}; for X=N atom, ~700 cm^{-1}. For methionine trans-trans form, 760 and 719 cm^{-1}; trans-gauch, 746 and 697 cm^{-1}; gauch-trans 667 cm^{-1}; gauch-gauch, 723 and 645 cm^{-1}.	43, 44
760	Trp, sharp and intense, easily recognizable; insensitive to microenvironment in the non-resonance Raman effect; In resonance Raman it may be quite sensitive to conformation changes.	31, 32
830 853	Tyr; Intensity ratio, I(853)/I(830) $\simeq$ 0.5 when Tyr is "buried" and involved in strong H-bonding (e.g., with carboxylate ion); the ratio increases to ~1.4 when Tyr is exposed to water.	61

Table 1 (continued)

Line (cm^{-1})	Interpretation	References
940	ν(C-C) due to Ile; side-chain conformation dependent.	unpublished results
1004 1014 1032 1209	Phe ring breathing mode Trp Phe Tyr	31,32
1220-1300	Amide III, 30% δ(N-H), 30% ν(C-N) in model compound N-methylacetamide; sensitive to secondary structure of polypeptides and proteins; α-helix: >1264 cm^{-1}, weak; β-structure: 1227-1240 cm^{-1}, strong; random-coil (solvated by H_2O): 1248 ± 4 cm^{-1}, board, medium intensity.	7, 8, 29, 30, 31, 32, 33, 34, 63
1361	Trp; sharp and intense in native lyozyme and α-lactalbumin but decrease in intensity upon denaturation or freeze-drying; sensitive to microenvironment; not well-understood at present.	7, 55
1410	N-deuterated His; sensitive to ionization state.	32
1420	symmetical - C stretching	31
1432	δ(N-H) of indole ring (Trp)	31
1448	δ(C-H);	31, 32
1550	Trp; Intensity may be quite sensitive to microenvironment in the resonance Raman effect.	31 and unpublished results
1530-1570	amide II; 60% δ(C-H); 40% ν(C-N) in N-methylacetamide; sensitive to secondary structure of polypeptides and proteins; weak in non-resonance Raman, strong in infrared and strong in resonance Raman, Trp line at 1550 cm^{-1} will not interfere with amide II if laser wavelength ~200 nm where Trp is out-of-resonance.	33, 34, 64 unpublished results
1630-1690	Amide I; ν(C=O); sensitive to secondary structure of polypeptides and proteins; α-helix: 1660 ± 4 cm^{-1}; β-structure: 1672 cm^{-1}, strong, sharp; random-coil (solvated): ~1655 cm^{-1} broad; Non H-bonded random-coil: ~1685 cm^{-1}.	29, 32, 54-64

Table 1 (continued)

Line (cm^{-1})	Interpretation	References
2500-2800	ν(S-H); sensitive to microenvironment; e.g., 2558 cm^{-1} (L-cysteine solid), 2583 cm^{-1} (L-cysteine solution at pH 5.2), 2560 cm^{-1} (hemoglobin), 2580 cm^{-1} (bovine and java lenses) 2570 cm^{-1} (turtle lens.)	56 and unpublished results

(~30-70% by weight) (Philipson, 1969; Kuck, 1970a). The lens is completely encapsulated and contains a single layer of more or less cuboidal epithelial cells at the front (anterior side), which transforms into lens fibers. Each fiber extends from anterior suture to posterior suture, having approximagely 10μ wide (Kuck, 1970a). There is a continuous growing of lens fibers throughout life, although the rate decreases with aging. As one proceeds from the cortex (outer portion) to the nucleus (center) one passes from protein synthesized recently to protein which was laid down in early life.

Laser Raman scattering is a non-destructive, sensitive and selective probe of lens structure at molecular level. In contrast to most biochemical methods requiring classical isolation procedures, it is used to obtain structural information directly from a living lens and thus avoids possible aerobic oxidation. The sulfhydryl groups which may be critical in the function of some lenses can be readily detecte d and measured quantitatively in the Raman effect (Yu and East, 1975). The question of whether the protein SH is directly or indirectly involved in the formation of cataract (or water-insoluble albuminoid) has been the subject of numerous investigations and considerable controversy (Testa et al. 1968; Pirie, 1968; Harding, 1970, 1972 a,b; Takemoto et al. 1974). Since the lens develops from ectodermal embryonic cells, from which the epithelium of the skin also originates, a parallel has been drawn between the keratinization involving loss of solubility of lens proteins that occur during the aging process. Testa et al. (1968) examined the SS/SH ratio in the water-insoluble albuminoid from different types of human cataract and found that SH fell and SS rose as the cataract became total so that the ratio was 0.87 in albuminoid of lenses having mainly cortical or nuclear cataracts and 5.2 in albuminoid from two total cataracts. In contrast, Pirie (1968) presented evidence that the interchain SS bonds detected were artifacts (formed during extract with water) and that none was present in the intact cataractous lens. More recently, Harding (1973) claimed that during cataractogenesis unfolded proteins accumulated in human lens, leading to formation of protein-glutathione and protein-protein disulfide bonds. Takemoto et al. (1975) studied the hereditary cataractous lens of Wistar rats by dissolving them anaerobically in 7M guanidine chloride solution to prevent air-oxidation and found that there was a significantly lower content of cysteine and significantly higher content of cystine in mature cataractous lens.

Very recently, we commenced a collaborative Raman study on aging effects in rat lens with Professor John F.R. Kuck of the Emory Ophthalmology Department. The precipitous drop of SH peak intensity at 2580 cm^{-1} with a concomitant increase of the S-S peak at 508 cm^{-1} provides an unequivocal evidence that the SS bonds do form in vivo in the process of aging (See Figures 1-2). The nature of the SS bonds (protein-glutathoine, inter- or intra chain S-S) remains to be determined by a combination of physical and biochemical methods. The rate of SH→SS conversion appears to be species-dependent and is linked to glucose metabolism (Auricchio and Testa, 1973). For example, the lenses of bovine (Yu and East, 1975) and Monkey (Figure 3) at about 3 years old display appreciable SH intensity in the nucleus. Auricchio and Testa have demonstrated that in vitro the lens of normal rabbit eyes is able to maintain the proteins in the reduced form even in the presence of high oxygen tension, so long as the capsule and the epithelium are apparently uninjured and glucose is available in the incubation medium. When the capsule is removed and glucose is not added, there is a fall of free glutathione and oxidation of water-soluble proteins. They also show that experimental "uveitis" causes a very early change in conformation of structural lens proteins, which makes them more reactive leading to formation of artifactual disulfide bonds during the preparation procedure. It should be of considerable interest to repeat the experiments employing a non-destructive method such as laser Raman scattering.

At present, little is known about the relationship between aging and human senile cataract. There is no satisfactory animal model for senile cataract. It is not clear why the albuminoid of rat lens is derived from γ-crystallin (Zigman and Lerman, 1965; Lerman, Zigman and Forbes, 1968), but that of bovine is from α-crystallin (Harding, 1969; Mok and Waley, 1967, 1968). A combination of Raman structural investigation and metabolic studies on older animals may provide new insight into the development of senile cataract.

Our Raman studies have been extended to include other vertebrate species such as chick and pigeon (Figure 3). We believe that comparison of the lenses from different species suggest a way of finding out more about the relationship between structure and function in lens proteins. In an earlier study from this laboratory (Yu and East, 1975), it was established that various crystallins of bovine (3 years old) exist in an antiparallel β structure (with a small amount ~15% of unordered structure) throughout the entire intact lens (proteins are believed to be globular). The most interesting finding we have made recently is that the lens protein in the nucleus of chick and pigeon lenses is exclusively α-helical structure, in sharp contrast to that of bovine or monkey (Fig. 3). The protein in the chick lens was designated as "FISC" (first important soluble crystallin) by Rabaey (1962), and referred to as δ-crystallin by Zwann (1966). The amino acid composition is known: Asp 7.2, Thr 6.6, Ser 8.4, Glu 14.7, Pro 2.9, Gly 6.0, Ala 7.6, Val 6.7, Met 1.8, Ile 7.4, Leu 13.3, Tyr 1.5, Phe 2.3, Lys 6.7, His 1.7, Arg 4.6 and Trp 0.7 moles % (Hoenders, 1965; Waley, 1969). Interestingly enough, it contains 63% of α formers (Glu, Ala, Leu, His, Met, Glu, Trp, Val, Phe, Ile and Lys). Although sequence of this unusual protein is unknown, it does have a high helix-forming potential, with $\langle P_\alpha \rangle \gg \langle P_\beta \rangle$ and $\langle P_\alpha \rangle = 1.10 > 1.03$ (a value required for helix formation) (Chou and

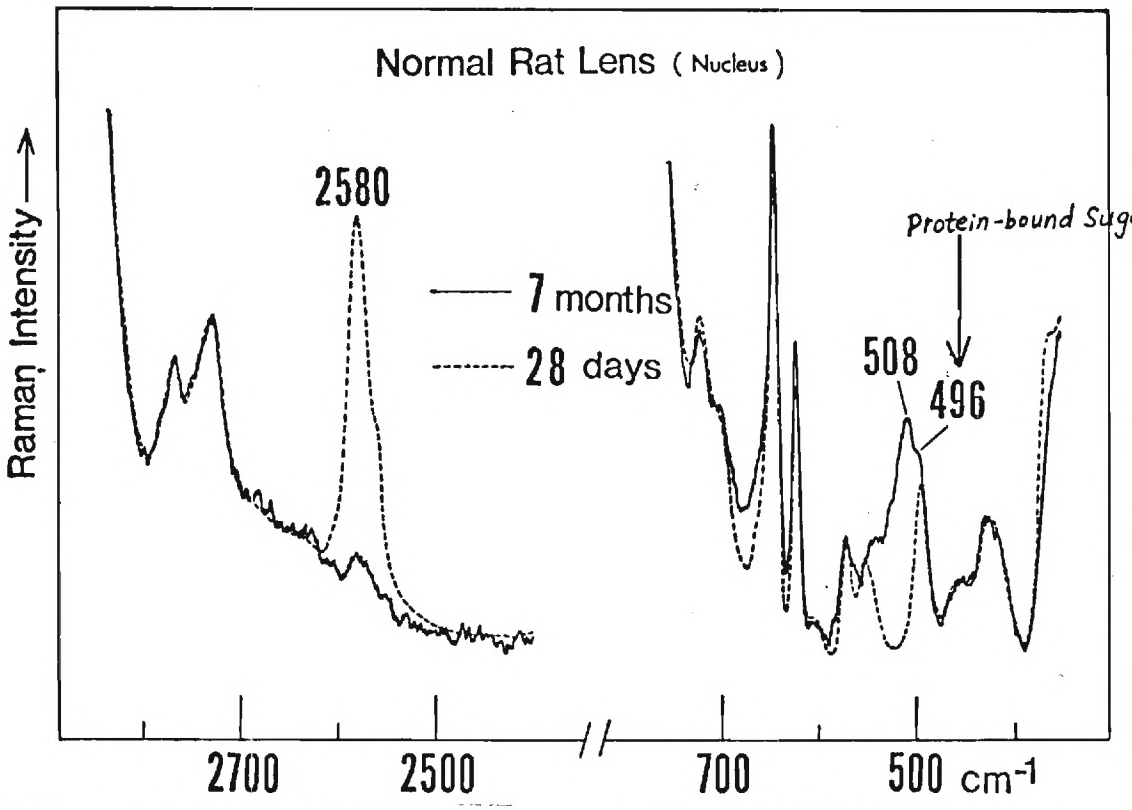
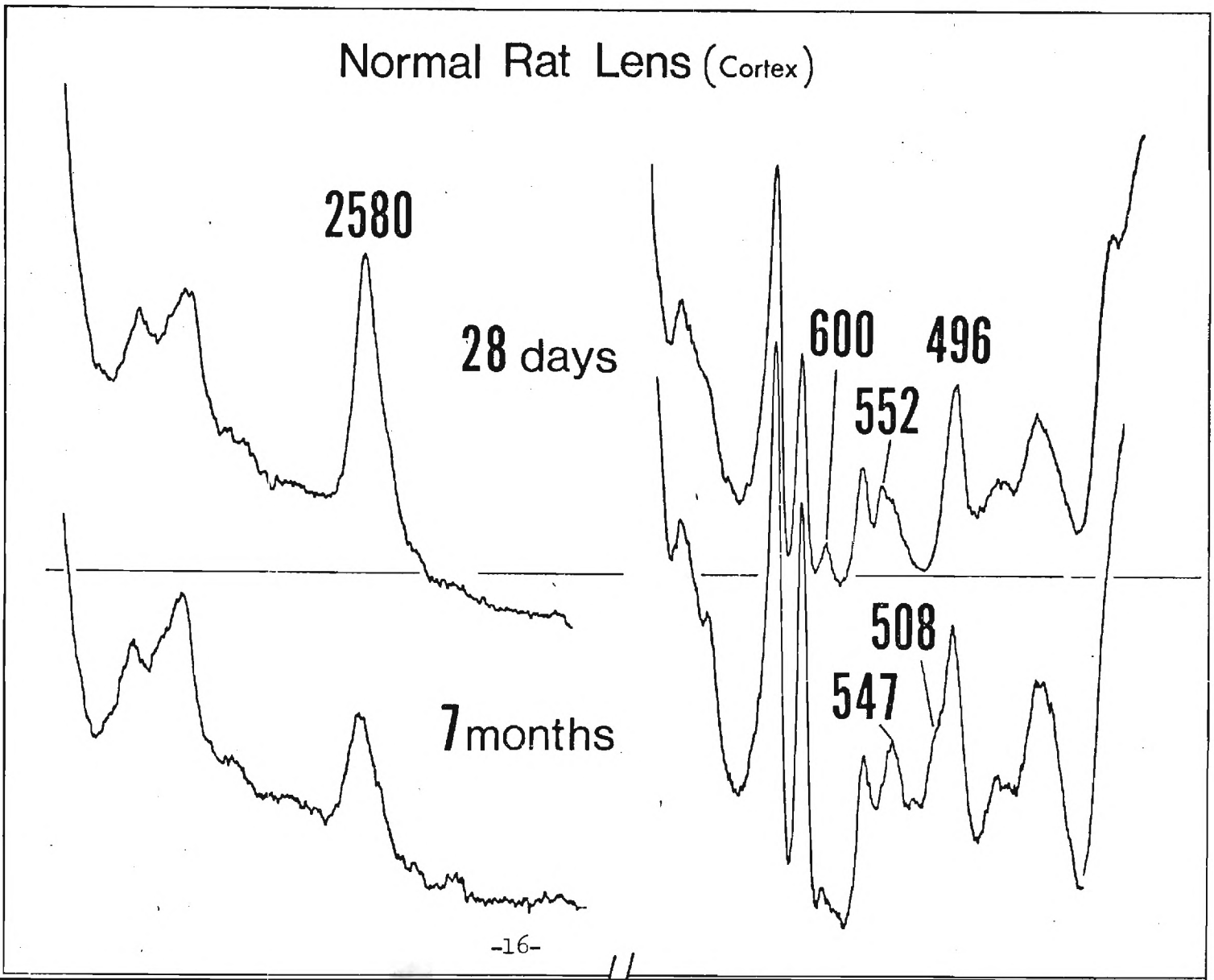


Figure 1

Figure 2



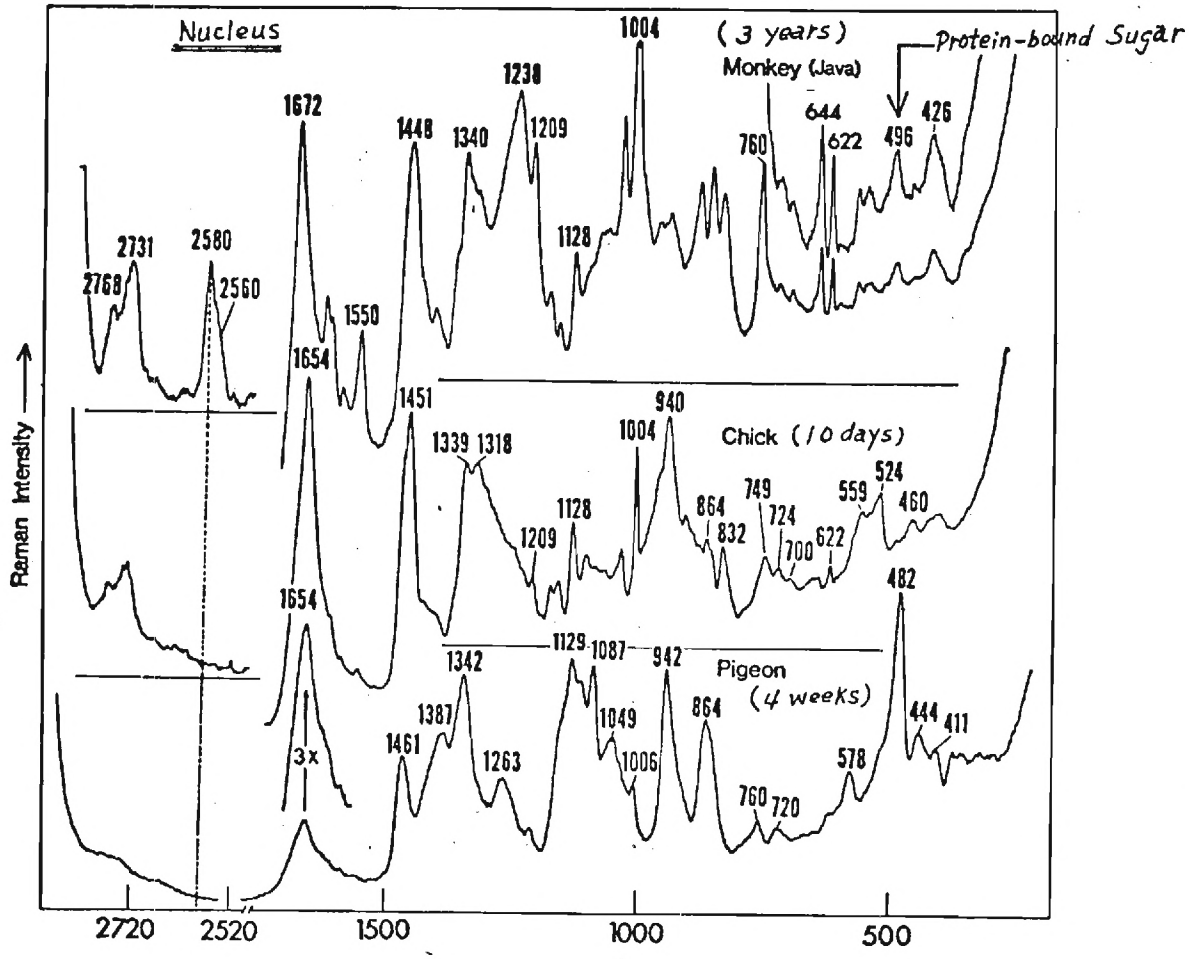

Nucleus
(3 years)
Monkey (Java)
Protein-bound Sugar
Chick (10 days)
Pigeon
(4 weeks)
Raman Intensity
3x
2720
2520
1500
1000
500

Figure 3

Fasman, 1974). Furthermore, the Raman spectra of both chick and pigeon lenses do not indicate the presence of protein-SH which might be correlated with the fact that birds are not subject to cataract with the same frequency as other animals (Kuck, 1974a,b). Since the lens of flying birds must accommodate rapidly and be assured of high acuity at all distances, it must be soft (80% water and no albuminoid) and deformable by a mechanism that is still not completely understood (Kuck, 1974a). At present, we are interested in determining by polarized Raman spectra (Fanconi et al., 1969) whether the α-helical polypeptide chains of δ-crystallin are oriented in a certain fashion either in the nuclear region or near the lens annular pad (Kleifeld, 1956). The spectrum of pigeon lens in Fig. 3 indicates high concentration of glycogen. A study on the topological distribution of this unusually high molecular weight carbohydrate in pigeon lens is of considerable interest.

The concentration of lenticular glutathione is estimated to be lower than 2×10^{-3}M in the nucleus of pigeon lens (Kuck, 1970a). Recently, we have been able to detect the presence of glutathione-SH in pigeon by UV resonance Raman technique. The pre-resonance enhancement ($\sim 10^3$) of Raman scattering due to -SH groups is obtained when the exciting radiation (2572.5Å in this case) is near the absorption region of the -SH chromophore (Tocelyn, 1972). The excitation radiation at 2572.5A was produced by doubling the frequency of the 514.5 nm line of the argon ion laser, with an Interactive Radiation model 5-110 UV generator (ADP crystal). The technological development of a pulsed UV tunable laser with a suitable technique for discrimination against fluorescence such as a gate detection system or coherent anti-Stokes Raman spectroscopy (Begley et al. 1974a,b) should make laser Raman technique an extremely powerful tool for lens research. It may be possible to probe into the detailed structure of RNA or DNA in a living epithelium cell, or the mechanism of the transformation of epithelial cells to lens fibers.

(2) The prediction of the Secondary Structure of γ-crystallin by the Method of Chou and Fasman.

The γ-crystallins are a group of low-molecular weight proteins present in most veterbrate lens. They are unusual in that they behave as cryoproteins. Interest has been centered on these proteins, as it has been suggested that they may be involved in cataract formation (Charlton and van Heyningen, 1968; Zigman, 1971).

The amino acid sequence of γ-crystallin (fraction II) from calf lens is known (Croft, 1972). The new predictive model of Chou and Fasman (1974) has been applied to the analysis of its secondary structure (see Fig. 4). When four helix formers out of six residues or three β-formes out of five residues are found clustered together in any native protein segment, the nucleation of these secondary structures begins and propagates in both directions until terminated by tetrapeptides breakers with 50% or more helix (or β-sheet) breaking or indifferent residues. Two rules have to be satisfied: (1) For a helical segment, $\langle P_\alpha \rangle \gg 1.03$ as well as $\langle P_\alpha \rangle \gg \langle P_\beta \rangle$; (2) For a β-sheet $\langle P_\beta \rangle \gg 1.05$ and $\langle P_\beta \rangle \gg \langle P_\alpha \rangle$. Here $\langle P_\alpha \rangle$ and $\langle P_\beta \rangle$ are average helical and β-sheet potential, respectively. The values for $\langle P_\alpha \rangle$ and $\langle P_\beta \rangle$ for a

given segment can be calculated according to Table 2.

From Fig. 4, it can been seen that the predicted structure contains extensive β-sheets, in complete agreement with our Raman results (Yu and East, 1975), excepting the helical segment (residues 121-127). It should be noted that although the segment (108-120) is predicted as β-sheet with $\langle P_\beta \rangle = 1.08$, it has an almost equal potential for helix formation (i.e., $\langle P_\alpha \rangle = 1.07$). Thus the transformation, α-helix $\rightleftharpoons$ β-sheet, involving this segment is quite possible with slight change in temperature, which might be related to the so-called "cold cataract" phenomenon in young animals (Zigman and Lerman, 1965).

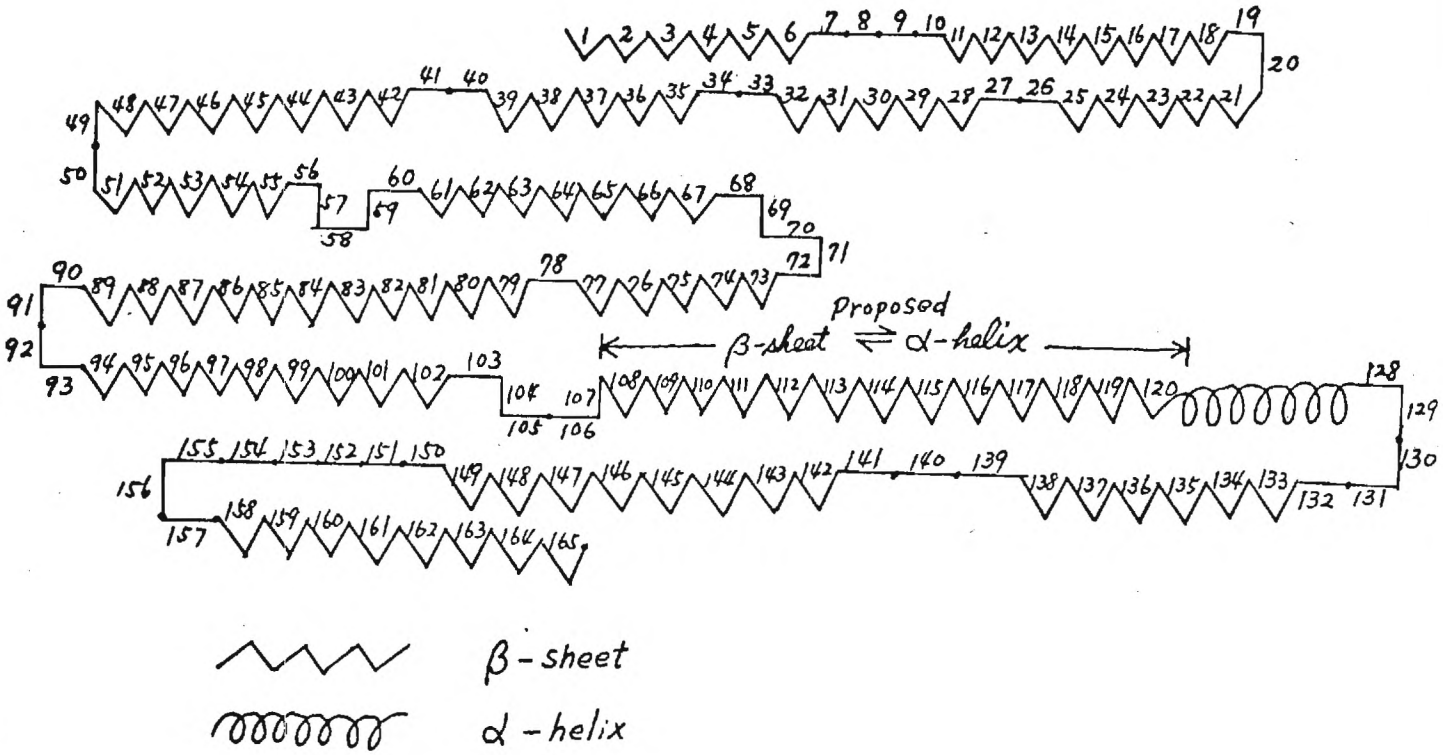


Figure 4

Table 2
(taken from Chou and Fasman, 1974)

Helical Residues	P_α	β-sheet Residues	P_β
Glu	1.53	Met	1.67
Ala	1.45	Val	1.65
Leu	1.34	Ile	1.60
His	1.24	Cys	1.30
Met	1.20	Tyr	1.29
Gln	1.17	Phe	1.28
Trp	1.14	Gln	1.23
Val	1.14	Leu	1.22
Phe	1.12	Thr	1.20
Lys	1.07	Trp	1.19
Ile	1.00	Ala	0.97
Asp	0.98	Arg	0.90
Thr	0.82	Gly	0.81
Ser	0.79	Asp	0.80
Arg	0.79	Lys	0.74
Cys	0.77	Ser	0.72
Asn	0.73	His	0.71
Tyr	0.61	Asn	0.65
Pro	0.59	Pro	0.62
Gly	0.53	Glu	0.26

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585-28-9345

Application of "CARS" Spectroscopy to the Studies of Pigmented Human Lens.

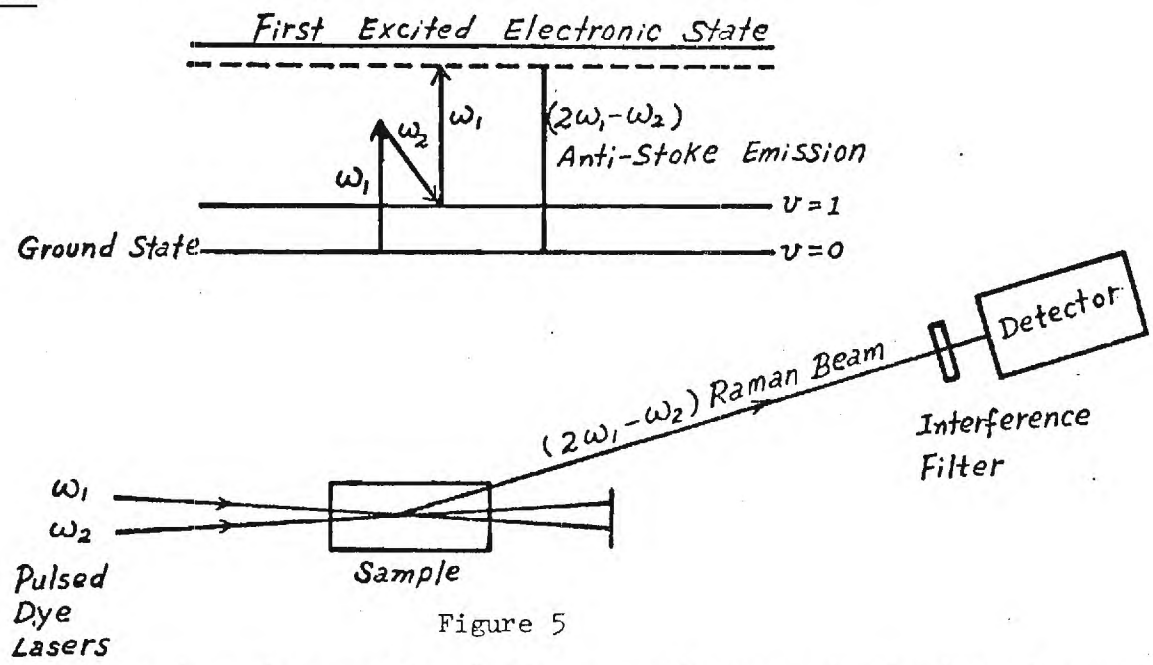


Figure 5

The most serious problem in Raman spectroscopy is the interference due to fluorescence, which can be caused by either fluorescent impurities or sample itself. The impurities usually can be removed by modern purification techniques. In the studies of colored intact human lenses, extraction of vibrational information unique to proteins and carbohydrates in the presence of high fluorescent background is a formidable problem. Although methods are available to suppress fluorescence background such as phase-detection with a rotating polarizer (Arguello et al., 1974), or pulsed laser excitation with a gate detection system (Yaney, 1972), they will fail to reduce the fluorescence in pigmented human lens since macromolecules are not rapidly rotating and the laser pulse duration (N_2 laser) is $\gtrsim$ 10 nsec. The fluorescent lifetime of those pigments (presumably trytophan derivaties) is ~2 nsec with a quantum yield of 0.2 (R. F. Borkman, private communication). Instead, we propose to utilize a novel technique, the so-called "CARS" spectroscopy (Begley et al., 1974; Hudson, 1974).

This technique makes use of Raman type resonance in the third order nonlinear optical polarizability. Usually the response of a medium to an electromagnetic field is written as

$$\vec{p}\,(\vec{r},\omega) = \chi(\omega)\,\vec{E}(\vec{r},\omega).....(2)$$

where $\vec{p}$ is the induced electronic polarization. $\vec{E}$ is the electric field, and χ is the electric susceptibility. The linear relationship between polarization and electric field is valid only when electric field is very small as is the case in the oridinary optics, but when electric field is very large one cannot use equa-

tion (1) and instead a power series expansion of polarization (Bloembergen, 1965):

$$\vec{p} = \chi^{(1)} \cdot \vec{E} + \chi^{(2)} : \vec{E}\vec{E} + \chi^{(3)} : \vec{E}\vec{E}\vec{E} + \ldots\ldots\ldots (2)$$

The coefficients $\chi^{(n)}$ are tensors of rank n+1 and called nonlinear susceptibility of n order.

When two light beams at frequencies ω_1 and ω_2 are incident on a nonlinear material, coherent emission at frequency $\omega_3 = 2\omega_1 - \omega_2$ is generated through the third order nonlinear polarization (see Fig. 5 for schematic of $(2\omega_1 - \omega_2)$ Technique). The third-order non-linear susceptibility $\chi^{(3)}$ associated with this polarization is responsible for the emission. The third-order nonlinear susceptibility has the form (Maker and Terhune, 1965):

$$\chi^{(3)} \alpha \frac{1}{(\omega_1 - \omega_a)(\omega_1 - \omega_2 - \omega_R + i\Gamma_R)(2\omega_1 - \omega_2 - \omega_b)} \qquad (3)$$

where ω_a and ω_b are electronic transition frequencies, ω_R is the Raman vibration frequency and Γ_R is the halfwidth at half maximum of the Raman signal. $\chi^{(3)}$ shows resonant behavior as $\omega_1 - \omega_2$ approaches ω_R and therefore produces the Raman signals.

This non-linear Raman technique has a greatly enhanced conversion efficiency compared to spontaneous Raman scattering and, in addition, it is a coherent beam. These effects lead to a greatly enhanced signal-to-noise ratio so that experiments which are impossible with conventional Raman technique become feasible through the CARS technique.

In CARS spectroscopy background fluorescence is practically non-existent since the signal is on the anti-Stokes side of the primary pump frequency while the fluorescence is on the Stokes side.

One serious disadvantage of this technique is that it requires relatively high (~10 w/w %) sample concentrations when water is the solvent (Begley et al, 1974b). However this condition is met in a human lens, which contains approx. 50 w/w % proteins. A preliminary CARS experiment on a bovine lens was carried out in Molectron's CARS laboratory in Sunnyvale, Calif. by Dr. G. K. Klauminzer (Molectron Corp.), Mr. S. Asher (Prof. K. Sauer's graduate student at UC Berkeley) and the Principal Investigator on Aug. 14, 1975. A strong coherent anti-Stoke Raman beam was actually observed when we set $\omega_1 - \omega_2 = 1004\ cm^{-1}$ (a ring-breathing mode of Phe in lens proteins). This enouraging results indicate that water content in ocular lenses is low enough to allow direct observation of Raman vibrational modes of proteins. The CARS spectrum of a pigment, diphenyloctatetraene in benzene was recently obtained by Dr. G. K. Klauminzer, B. S. Hudson and I. Chabay, and published in a Molectron's application note.

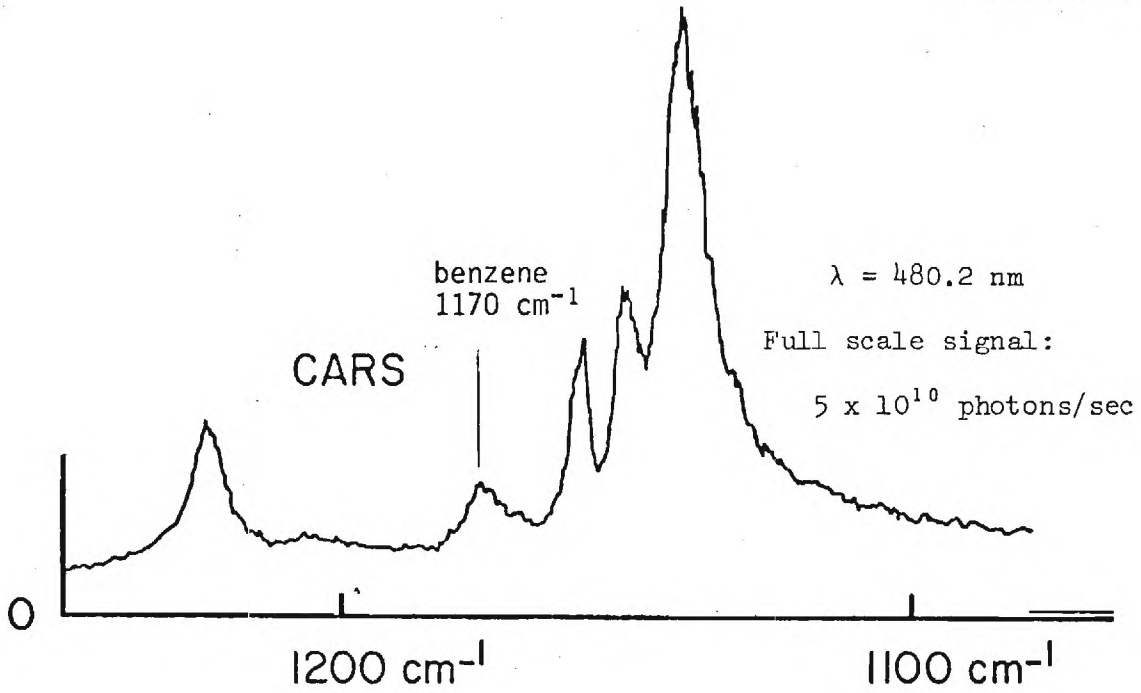


Figure 6 CARS spectrum of diphenyloctatetraene
1.4×10^{-3}M in benzene) (Courtsey of Molectron Corp.)

3. Rationale

Laser Raman spectroscopy is an ideal probe of lens structure at molecular level. It is non-destructive and thus the information obtained is reliable. When one enters into UV resonance Raman scattering region, the technique becomes highly sensitive (10^3 - 10^4 enhancement) and selective.

B. Specific Aims

This proposed research has the following specific objectives:

(1) To establish the structural similarities and differences between animal models and the human lens.
(2) To select and develop suitable animal models for human senile cataract.
(3) To identify the nature of lens protein alterations in relation to aging, evolution and cataract formation.
(4) To explain why birds and cats have low incidence of cataract.
(5) To learn something about the mechanism of lens development (the transformation of epithelial cells to lens fibers).

C. Methods of Procedures

This research combines our competence as spectroscopists with the ophthalmic competence of the staff of Emory University Medical School. In general, human and animal lenses (normal and in various pathological states related to cataractogenesis) will be provided by Professor Kuck (see attached letter of support).

As mentioned in the section of Introduction, the vertebrate species under investigation will tentatively include: Amphibia (frog), Reptiles (snake, turtle), Birds (pigeon, chicken, duck, turkey), Fishes (dogfish, trout, lamprey), and Mammals (Java, bovine, rabbit, guinea pig, rat, whale, bat, cat, dog). Other species will be included if necessary.

The estimation of the age of guinea pigs, rabbits and chickens will be made by means of determining their lens weight (Hackwin, 1971). The relationship between age and lens weight for rats has been carefully determined in Prof. Kuck's laboratory. For other species, the lens weights will be recorded prior to Raman scattering experiments, and the ages estimated from the information provided by the sources.

The intact lenses will be maintained in a modified KT medium (Thoft and Kinoshita, 1965). We use a 3-part medium which is mixed just before use. Part I: $MgSO_4$ (55.6 mg/500ml), Na_2HPO_4 (34.3 mg/500ml), KH_2PO_4 (34.3 mg/500ml), KCl (248.0 mg/500mg), $KHCO_3$ (84.0 mg/500ml) and $NaHCO_3$ (1.94 g/500ml); Part II: NaCl(7.90 g/500ml); Part III: $CaCl_2$ (1.7 g/100ml). Add equal volumes of I and II and then add 1% of Part III. In addition, 140 mg ABGG mixture per 100 ml solution is added. The ABGG mixture (Antibiotic-Glucose-Glutamine) contains 4.0g glucose, 63mg penicillin G (K-salt), 63 mg streptomycin, and 0.45g glutamine. The mixture is gassed with 95% O_2-5% CO_2.

The phenomenon of "cold cataract" was recently re-investigated by Tanaka and Benedek (1975) using the technique of optical mixing spectroscopy. They concluded that it is the manifestation of a first-order phase separation of the protein-water mixture in the lens into separate co-existing phases. The cold cataract appears at the temperature at which the mean diffusivity, $\bar{D}$, of the solution reaches zero. Present evidence indicates that the cryoprotein in the rat lens is chiefly γ-crystallin (Zigman and Lerman, 1965). Although it was suggested that the phenomenon might involve a change in γ-crystallin configuration induced by low temperature (Kuck, 1974a), no experimental evidence has been presented. If the proposed "α-helix $\rightleftharpoons$ β-sheet" transition in the segment (108-120) of γ-crystallin is correct, the spectral change in the amide III region should be detectable.

Delta (δ) crystallin is presumably very prominent in the avian embryonic lens. Little is known about the structure and chemistry of this important protein. Quite likely, it was introduced into birds' lenses for special functional purposes during latter state of evolution. We plan to isolate this protein according to Hoenders (1965) and Watanabe and Kawakami (1973). Its properties and conformational behavior will be carefully investigated by laser Raman technique. Our recent finding (from Raman spectra) indicates that it is exclusively in the α-helical conformation in intact chick and pigeon lenses. It is of interest to determine if this crystallin remains in the α-helical structure in the isolated state (purified sample dissolved in aqueous solution). We also plan to determine the effects of aging process on protein structure in birds lenses.

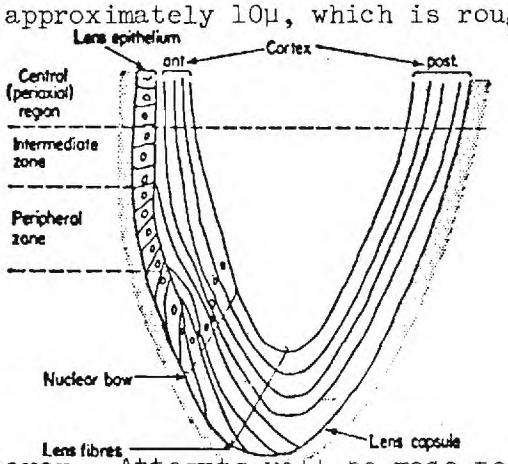
There has been indication (based on our Raman spectra of 10 years old hen's lens) that bird's lenses are quite heterogeneous in

secondary structure throughout the entire lens, in contrast to bovine lens (Yu and East, 1975).

In order to support our Raman spectroscopic finding on the conformation of δ-crystallin, we will carry out detailed optical rotatory dispersion (ORD) and circular dichroism (CD) studies of isolated δ-crystallin.

It should be noted that δ-crystallin of chick lens (see Fig. 3) does not contain a Raman signal at 496 cm^{-1}, which is now known to be due to protein-bound sugar (unpublished results). We have accumulated evidence that the sugar moiety is covalently bound to the protein. We hope to observe intensity changes in this region in the spectra of bovine lens as a function of ages, since there has been suggestion that sugar may involve in the aggregation of the subunits of α-crystallin (Spector et al., 1971) and that the insoluble albuminoid (mainly α-crystallin) of bovine lens increases with aging. It is also of interest to examine the role of sugar in the formation of albuminoid (chiefly γ-crystallin) in rats. This 496 cm^{-1} line appears quite strongly in the spectrum of γ-crystallin (Yu and East, 1975), consistent with the fact that bovine γ-crystallin has the highest level of bound sugar among various crystallins (Kabasawa and Kinoshita, 1973).

Experimentally, it is a challenging problem to study lens epithelium by laser Raman scattering because of its complexity in constituents. It is possible to reduce (by focusing) laser beam diameter to approximately 10μ, which is roughly the thickness of the



epithelium layer. Attempts will be made to obtain Raman spectra (non-resonance and resonance) of central region, intermediate zone, peripheral zone and nuclear bow. Comparisons of these spectra with that of lens fibers should be quite interesting.

Non-resonance Raman spectra of unpigmented human lens (ages 0-5) can be obtained using the existing facilities. As discussed in the Background section, Raman studies of pigmented older human lens require coherent anti-Stokes Raman technique. We have put about $40,000 in Equipment budget for purchasing UV pulsed dye lasers.

D. Significance

The work on structural similarities and differences between human and animal lenses will assist in evaluating the applicability of information obtained from animal lenses to human cataract, since a great deal of research has been carried out on animal models because of the limited availability of normal human lenses and the impossibility of experimentation on humans. Our special emphasis on older animal studies may provide new insight into the development of senile human cataract.

E. Facilities Available

Georgia Tech is adequately equipped for biological applications of laser Raman spectroscopy. We have two research-grade Raman spectrometers (Spex's systems), two argon-ion lasers (CR-3), a krypton ion laser (CR-500K), two dye lasers (CR-490A and SP-370), a lock-in amplifier (PAR 121), a frequency doubler (Interactive Radiation model 5-110 UV generator), a PAR Dual Channel Boxcar Integrator (model 162/97/99), two Gated Integrators (PAR-164) with 10 nsec-5msec Interval, and a PDP 8/f computer. Biochemical instruments include a scintillation counter, a preparative centrifuge, a LKB ReCychrome Chromatography system, a UVicord II flow analyzer, a UltroRac 700 fraction collector, and a Canalco PREP-DISC electrophoresis apparatus. Other items of equipment available in the Department include: Radiometer titrator with recorder; expanded-scale pH meter, temperature-jump apparatus, fluorescence-phosphorescence.lifetime measuring system, UV visible, IR, ESR, Fourier Transform NMR, ORD, MCD and CD spectrometers.

The Chemistry Department here maintains excellent machine, electronic and glass blowing shops. The Georgia Tech Price Gilbert Memorial Library has outstanding collections of scientific and technical publications.

Dr. Kuck's laboratory at Emory University is equipped to make related biochemical studies on lenses to supplement the results from Raman spectroscopy. Larger pieces of equipment available there are: Turner flucrometer, Advanced Instruments osmometer, custom-made UV irradiation bath, New Brunswick gyratory shaker bath, Gilson fraction collector and UV monitor, Varian gas chromatograph, photographic equipment. There is also the usual complement of water baths, ovens, centrifuges, balances, microscope, pH meter etc. Adequate animal quarters are maintained at the Eye Research Lab. including a colony of rats for aging studies and a colony of mice which appears to have some tendency for senile cataract. The medical library at Emory nicely complements the Georgia Tech library for the subject area of this application. Dr. Kuck has good contacts for the procurement of unusual lens species. Equipment is available for the in vivo UV irradiation of small animals for protracted periods of time. In addition, Dr. Kuck has access to over-age eyes from the eye bank and cataracts from operations performed at Emory University Hospital.

F. Principal Investigator Assurance

The undersigned agrees to accept responsibility for the scientific and technical conduct of the research project and for provision of required progress reports if a grant is awarded as the result of this application.

Aug. 18, 1975
Date

Nai-Teng Yu
Principal Investigator

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SCHOOL OF MEDICINE

DEPARTMENT OF OPHTHALMOLOGY
LABORATORY FOR OPHTHALMIC RESEARCH

August 12, 1975

Dr. Nai-Teng Yu
Department of Chemistry
Georgia Institute of Technology
Atlanta, GA 30332

Dear Dr. Yu:

This letter is to confirm my eagerness to continue our collaboration on research in the biochemistry of the lens. As in the past, I intend to supply you with lens material of interest to us and to carry out such procedures as glutathione analysis and protein separations. Also the aging animals in my rat and mouse colonies are at your disposal. And of course I will continue to act as your consultant in ophthalmology in giving advice and in procuring human lenses and cataracts.

Yours sincerely,

John F.R. Kuck, Jr. PhD
Associate Professor of
Ophthalmology and
Assistant Professor of
Biochemistry