RESONANCE-ENHANCED RAMAN SPECTRA OF VISUAL PIGMENTS IN INTACT BOVINE RETINAS AT LOW TEMPERATURES

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SUMMARY

Raman scattering of 4880 Å laser radiation from intact dark adapted bovine retinas, in the temperature range of -70 to -85°C, is reported. The observed spectra are contributed only by the visual pigments. The strongest line, at 1555 cm^{-1} is contributed by the ethylenic C = C stretching mode of retinal. Results on Raman spectra of the model system retinylidene hexylamine in acidified ethanol solution show this mode at 1560 cm^{-1}, supporting the hypothesis that the pigment in the retina is bound as a protonated Schiff base.

We report the detection of Resonance-enhanced vibrational Raman (RR) scattering from whole bovine retinas at temperatures between -85°C and -70°C. Assignment of the observed mode to bound visual pigments is proposed, and the state of protonation of the bound pigment, implied by reference to model systems, is discussed.

Resonance enhancement in Raman spectra may be attained when the excitation frequency is close to an absorption peak of the scatterer.[1] The effect is very pronounced in carotenoids and vitamins A[2] and the shape of the corresponding excitation profiles[3] makes possible the selective observation of a particular pigment in a complicated system such as an intact tissue.[4] Our experience with carotenoids in various plant tissues indicates that the Raman spectrum is completely dominated by its resonance enhanced constituent while the spectrum of all the non-resonant ingredients is attenuated by the absorbing pigment. The vibrational modes involving the skeletal C = C, C - C and C = N stretching deformations, which most effectively modulate the chromophor, are the only ones to be resonance-enhanced. The RR spectra of carotenoids and vitamins A respond to factors important to the biochemistry of vision: a) delocalization of π electrons, which weakens the C = C bonds,[5] b) stereoisomerism,[6] and c) formation of C = N bonds.[7]

The samples were dark-adapted bovine retinas shipped in dry ice by Hormel, Inc. The still frozen agglomerate of retinas was sawed in the dark into coin sized slices which were not processed any further. A slice was placed in a temperature-controlled cell ($T \geq 170^{\circ}K$) built for grazing-angle Raman spectroscopy.[8] Spectra were taken in the 1500-1600 cm^{-1} range, where the most intense resonance enhanced modes of conjugated linear molecules (C = C stretch) are located.

The output beam of a continuous-wave Argon laser at power levels of 30 mW or less was directed to graze the surface of the sample. The light scattered at $\sim 90^{\circ}$ was analyzed by a scanning double monochromator adjusted to ~ 6 cm^{-1} resolution. The detector was a photon counter with a digital data handling system, and had a practically unlimited dynamic range. This feature allowed the detection of Raman spectra superimposed on top of intense fluorescence bands. It took ten minutes on the average before the background of fluorescence[9,10] and of elastic scattering (caused by the particulate nature of the sample) stabilized to a degree that reproducible spectra could be obtained.

Considering the history of the sample (dark adapted, stored in dry ice, inserted into a cold ($-70^{\circ}C$) cell before being exposed to light) as well as the prevalence of visual rods in the retina, our Raman spectrum is most probably contributed by lumirhodopsin ($\lambda_{max} = 497$ nm).[11-13] [Correspondence between the low-temperature bleaching cycles in whole retina and in its frozen extracts is assumed.]

The conclusions that the scattered radiation has the resonance-enhanced Raman (RR) characteristics, that it is contributed by the visual pigments alone and then only by the C = C stretching mode, are based on the following evidence: a) The sideband nature of the Raman spectrum is ascertained by varying the wavelength of excitation (4880, 4765, 4579 Å) (Fig. 1). The shape of the spectrum does depend, however, on the wavelength. The strongest line in the spectrum is at 1555 cm^{-1}; at the longer excitation wavelength it shows a shoulder at 1520 cm^{-1}, which becomes progressively sharper and better resolved as

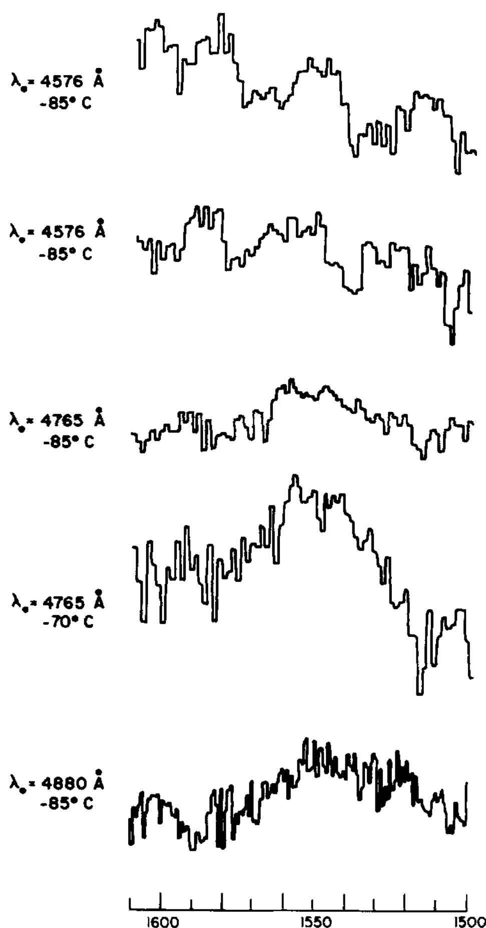

Fig. 1 Typical grazing incidence Raman spectra of frozen whole bovine retinas. The scan rate, limited by background drift, is 20 cm^{-1}/min. Integration time - ~ 3 sec. The two top traces, taken at different times on the same sample, indicate a reproducible spectrum in spite of drifting background. The major peak at 1550 cm^{-1} and the subsidiary peak at 1520, which shows as a shoulder in the spectra excited at longer wavelength, are definitely reproducible and therefore identified as Raman lines.

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the excitation wavelength is decreased from 4880 Å to 4576 Å. The shoulder may either differ from the main line in its excitation profile, or the two lines may be contributed by different bleaching products, the relative abundance of which depends on the excitation wavelength [see also Fig. 2]. Alternatively, the 1520 line may pertain to a macular carotenoid. b) The absorption peaks of lumirhodopsin, or for that matter of all the low temperature bleaching

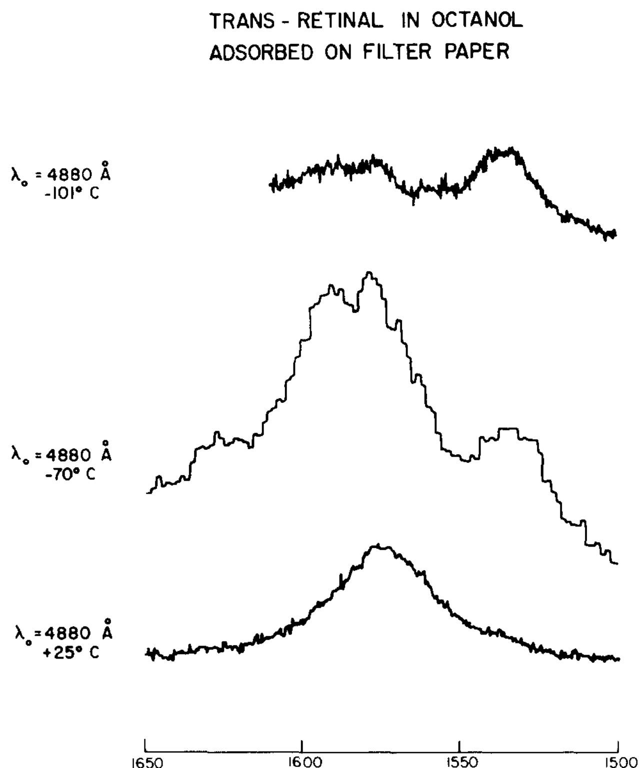

Fig. 2 Raman spectra of trans retinal, 1% solution in octanol, adsorbed on filter paper; grazing incidence of laser light at λ_o = 4880 Å. Scan speed 20 cm^{-1}/min.

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products of retinal chromoproteins[11-13] are close to the excitation wavelengths employed in the present experiment. Accordingly the Raman spectra of the pigments should be resonance-enhanced and dominant. The trend of resonance enhancement was indeed apparent even in dilute solutions of retinol and retinal excited in the red (6328 Å).[6] c) Under equal experimental conditions, no Raman signal was obtained from whole blood or dense protein suspensions.

Excitation at wavelengths close to the absorption peak of retinal is expected to isomerize the molecules. Indeed, the Raman spectra of 9-cis, 13-cis and trans retinal in hexane and octanol solutions indicated complete cis-trans conversion induced by the Argon laser light, while no conversion was caused by the red light of the He-Ne laser.[7]

The model systems were the neutral and the acidified solutions of trans-retinal and trans-retinylidene hexylamine in hexane, ethanol and octanol. The pertinent results (at room temperature) are: 1) The major transition of trans-retinal is at 1574 cm^{-1} in ethanol and at 1570 cm^{-1} in octanol. [The spectrum in octanol was run at low temperatures (Fig. 2), where several peaks were observed. The major peak in the -70°C spectrum is the only one seen at room temperature.] 2) The major Raman line of trans-retinylidene hexylamine is at 1582 cm^{-1} in ethanol and at 1584 in hexane. Upon acidification, this mode shifts down to 1560 cm^{-1} in both solvents. 3) In acidified ethanol solutions of retinal the C = C line shifts all the way down to 1530 cm^{-1}.

The major peak in the spectrum of the whole retina is at 1555 cm^{-1} (Fig. 1), measurably lower in frequency than the major line in either free retinal or in the normal (basic) Schiff base. Although one cannot conclude from the proximity of this frequency to that of the C = C line in the <u>protonated</u> Schiff base that a $-C{=}\overset{+}{N}H-$ bond bridges the chromophor to the lipoprotein in the bleaching product under observation (predominantly lumirhodopsin), such a model[14-18] is supported by our finding.

Our results may have relevance to retinal therapy by Argon ion laser irradiation. Raman scattering by visual pigments probably is a by-product of the therapy, and as such may deserve collection and sampling.

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