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FUNDAMENTAL STUDIES IN THE MOLECULAR BASIS OF LASER
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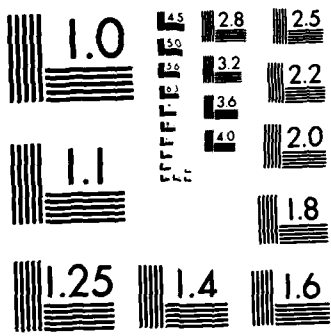
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Fundamental Studies in the Molecular
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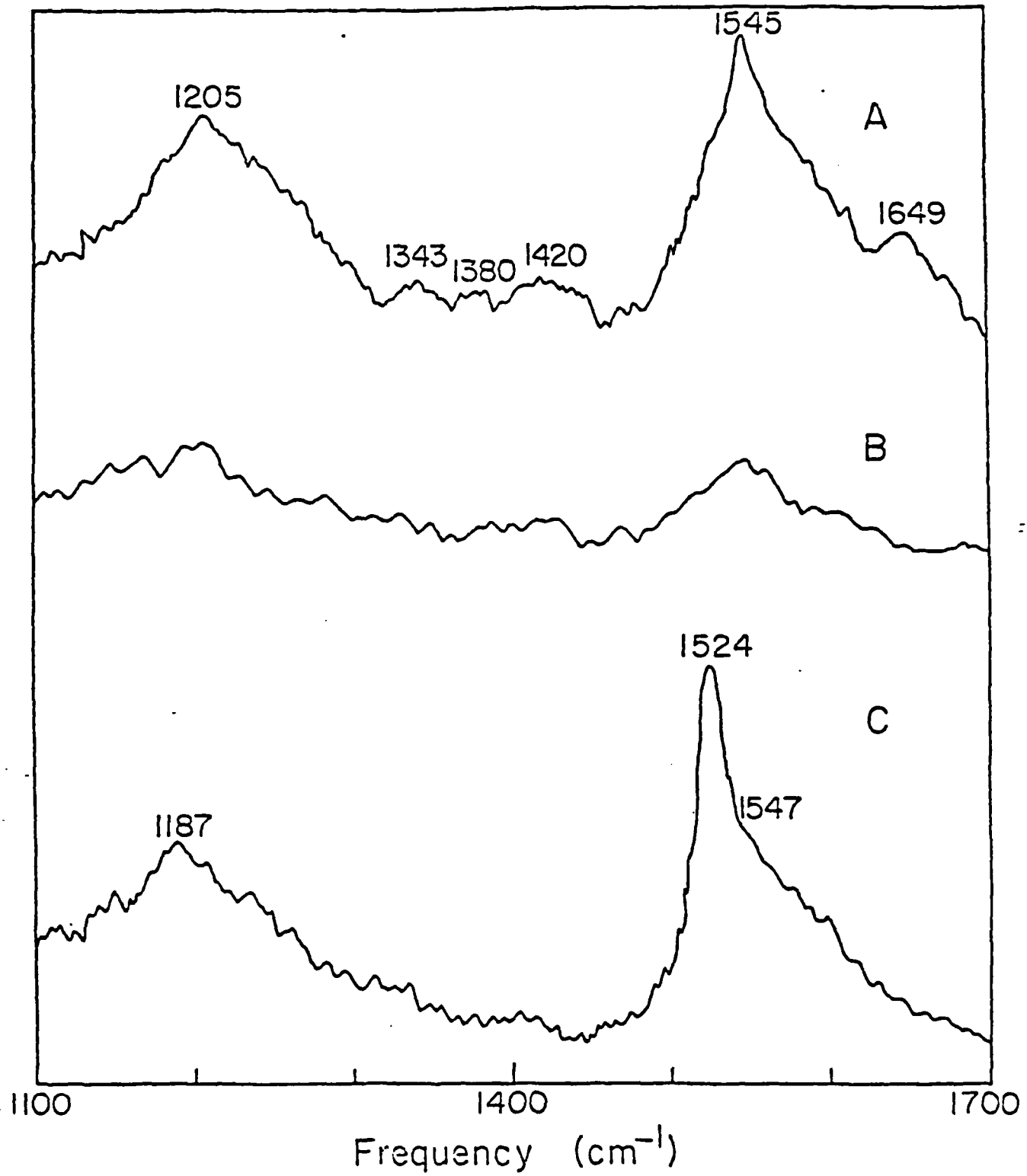
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Tunable laser resonance Raman spectroscopy was one of the tunable laser techniques we proposed to apply to investigate the molecular basis of retinal damage problems. As we discussed in last year's proposal, a principal feature of this technique is the capability to selectively enhance with appropriate tunable laser frequencies the structurally and environmentally sensitive vibrational spectra of various components of photoreceptor cells. One of the most attractive experimental features of tunable laser resonance Raman spectroscopy is the flexibility which allows this spectroscopic tool to study any form of matter from single crystals to dilute solutions to whole tissue. In essence, all that is needed to obtain the selective enhancement of the vibrational Raman spectrum of a chromophore embedded in a tissue is to scatter a laser off the tissue at an appropriate frequency within the electronic absorption of the chromophore and focus the scattered radiation into a monochromator. However, as we mentioned in last year's proposal, a principal problem in Raman spectroscopy is fluorescence which occurs at longer wavelengths (lower energies) than the incident laser wavelength. This is the same region where the strongest Raman scattering is detected, and since fluorescence is more intense than Raman scattering it usually obliterates the Raman spectrum when it occurs.

In photoreceptor cell preparations the principal fluorescence problem arises from accessory pigments in the pigment epithelium. This, of course, has precluded in the past in vivo experiments in normal pigmented eyes, and we have therefore had to resort to albinos to apply this technique in vivo. However, as we mentioned in last year's proposal, recently, significant progress has been made in rejecting fluorescence from interfering with the Raman spectra. This progress is based on a fundamental difference between Raman scattering and fluorescence. This difference is simply the fact that fluorescence is observed at the same spectral frequencies no matter what the incident laser excitation, whereas the Raman scattering frequencies are tied to the incident laser frequency. We proposed last year to apply such an innovation to pigmented eyes, since only then could the technique be applied with all its power to retinal damage problems at the molecular level.

With this in mind, we have expended a significant effort during the past year to apply these new ideas in vivo in pigmented eyes, and I am happy to report that we have been quite successful.

The results we obtained in our initial experiments with pigmented bovine eyes are seen in Figure 1. The data in Figure 1A were obtained with a laser flash centered around 614 nm of 1μsec duration with a repetition rate of 30/sec and an average power of 1 mW. We see no interference from the fluorescence that usually plagues experiments in such pigmented



eyes. This spectrum arises in all probability from the retinylidene chromophore of rhodopsin. This is understandable since the laser is in resonance with that molecular entity in the eye. To prove this hypothesis dramatically, we changed the laser's central frequency to 640 nm and the spectrum disappears (Figure 1B). Bleaching the eye causes a significantly different spectrum obtained under the same conditions but with the laser at 488 nm (center frequency). The spectrum of the bleached eye probably is from retinol.

These developments for the first time open up new avenues to examine the molecular changes of rhodopsin, melanin and other components under the exact conditions of retinal damage.

In addition to the progress described above, we have been making steady progress in our attempts to investigate the possible role of phosphodiesterase (PDE) and GTPase in the unique damage mechanisms discovered by Beatrice and co-workers. Using the unique characteristics of lasers and recent developments in microfabrication techniques, we proposed to develop a new form of light microscopy with angstrom resolution (ARLM) to investigate the roles of these newly-discovered, light-activated enzymes. For this project the Medical Research and Development Command allotted \$35,000 as a supplement to my contract for the purchase of equipment for this project. The equipment for the experiments have been designed and orders have been sent out for the equipment. In addition, I have been actively engaged in trying to obtain antibodies for these enzymes which will be essential for the full application of

ARLM to photoreceptor damage mechanisms. As a first step we have been purifying phosphodiesterase (PDE). As a result of irreversible binding of bovine phosphodiesterase to conventional ion exchange resins, we have fractionated EDTA solubilized PDE by velocity centrifugation through a 5-20 percent sucrose gradient and purified it by batch ion-exchange chromatography on polyhistidine agarose. The results for bovine PDE have been very good giving high activity relative to unpurified photoreceptor cells. We have been using this purified PDE to get rabbits to form antibodies which can then be fluorescently labelled. These labelled antibodies, together with labelled antibodies for rhodopsin, will be used with ARLM to study the localization and change in localization of PDE relative to rhodopsin as a function of damage.

Finally, a very exciting result has been the extraction of rhodopsin as a function of laser irradiation of bovine retina. The results we have obtained are listed in the following table:

Laser Wavelength	Laser Power	Apparent Rhodopsin Molecular Weight
	0	37,500
514.5 nm	1 mW	39,500
514.5 nm	2 mW	43,250
514.5 nm	4 mW	46,000
514.5 nm	8 mW	54,000
514.5 nm	10 mW	60,000
514.5 nm	12 mW	61,000
514.5 nm	14 mW	62,000

Laser Wavelength	Laser Power	Apparent Rhodopsin Molecular Weight
514.5 nm	16 mW	62,250
514.5 nm	18 mW	62,250
514.5 nm	20 mW	62,250

All of these results were obtained with c.w. laser irradiation. The data clearly show that there is definite aggregation of rhodopsin with other rhodopsins or with other proteins in the cell for these irradiation and wavelength conditions. We are in the process of getting data for other radiation conditions.

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