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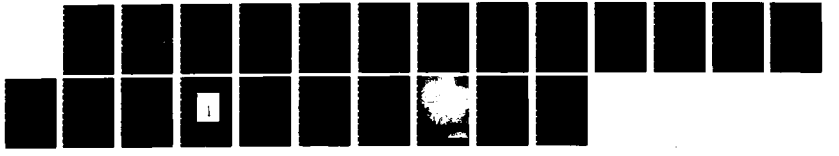

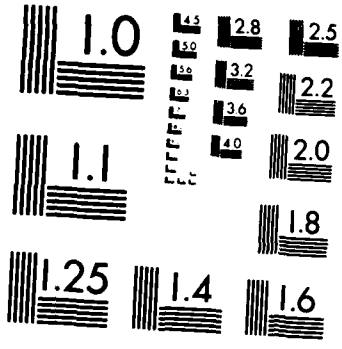

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Fundamental Studies in the Molecular Basis of Laser Induced Retinal Damage

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As a result of the recommendations of the Vision and Laser Bioeffects Subcommittee and extensive discussion with Col. Beatrice and Dr. Harry Zwick our laser spectroscopy experiments have focused on cones in the red-eared swamp turtle, Pseudemys scripta elegans. Our choice of this system was based on the desire to correlate our molecular data with the extensive data being collected on this system by Dr. Zwick in his studies on laser hazards at LAIR. Thus, a detailed collaborative effort was initiated on this system during the past year after our contract was awarded in February. The experiments have already resulted in important information which opens new avenues to explore fundamental molecular mechanisms of retinal damage with laser irradiation. A part of these significant new results have already been submitted for publication to Nature and will be summarized below in Section I.

The results of these experiments will be summarized in the following sections:

I. Elucidation of the role of oil droplets in the absorption of light by the turtle retina.

The most prominent aspect of turtle retina is the cone oil droplets. The only physical studies completed on these organelles were absorption measurements and even these measurements were unable to be performed on the intact oil droplets since their absorption was flat with a long wavelength cutoff (1). However, finally, absorption measurements were made on structurally altered oil droplets swollen by 1000 times with mineral oil (2). This investigation obtained absorption spectra which were interpreted with the help of biochemical extraction data (3) to be somewhat similar to carotenoids but with an absorbance in the blue which was incompatible with that of carotenoids. Using the techniques of tunable laser resonance Raman spectroscopy described in our earlier proposal, we have obtained detailed structural data on intact oil droplet in suspension and intact on the retina. These studies have not only identified the specific molecular species in the oil droplets but have also elucidated a detailed molec-

ular explanation for the origin of the absorption spectra of these organelles which leads to important new suggestions for the participation of these oil droplets in damage mechanisms.

Before discussing the data, let me remind the reader that the technique we have used, resonance Raman spectroscopy, is a unique tunable laser tool. This spectroscopic technique involves choosing a tunable laser wavelength within the absorption of a particular rod or cone pigment and then scattering the laser off the retina. In the scattered light there will be components with wavelengths which are greater than the incident laser wavelength. This component is directly related to the vibrational spectrum of the chromophore involved in the absorption. Specifically, then, by choosing a laser frequency within the absorption of a specific chromophore one is able to detect selectively the structurally and environmentally sensitive vibrational spectrum enhanced above the background of surrounding protein, lipid and other components in the cell. This vibrational spectrum has been shown in our previous studies to be very sensitive to even the most subtle structural alterations.

Since resonance Raman spectroscopy is a scattering process the high optical density of the oil droplets, which seriously perturbed the absorption measurements, do not complicate the Raman process. Thus resonance Raman data with excellent signal to noise ratio have been obtained as is seen in Figure 1. The resonance Raman spectrometer used for this measurement was equipped with a tunable laser so that exciting wavelengths within the cone pigment absorptions, between 450 nm and 570 nm, were available. In order for maximum overlap in the experimental procedures, the retinal preparations have been obtained from turtles disected at LAIR by Harry Zwick and Steve Schuschereba. The spectrum in Figure 1 was obtained with 520.8 nm excitation. The bands seen in the spectrum can be definitively assigned to carotenoids based on the work of Lewis et al (4). In addition the higher resolution spectrum in Figure 2 shows a weak band at

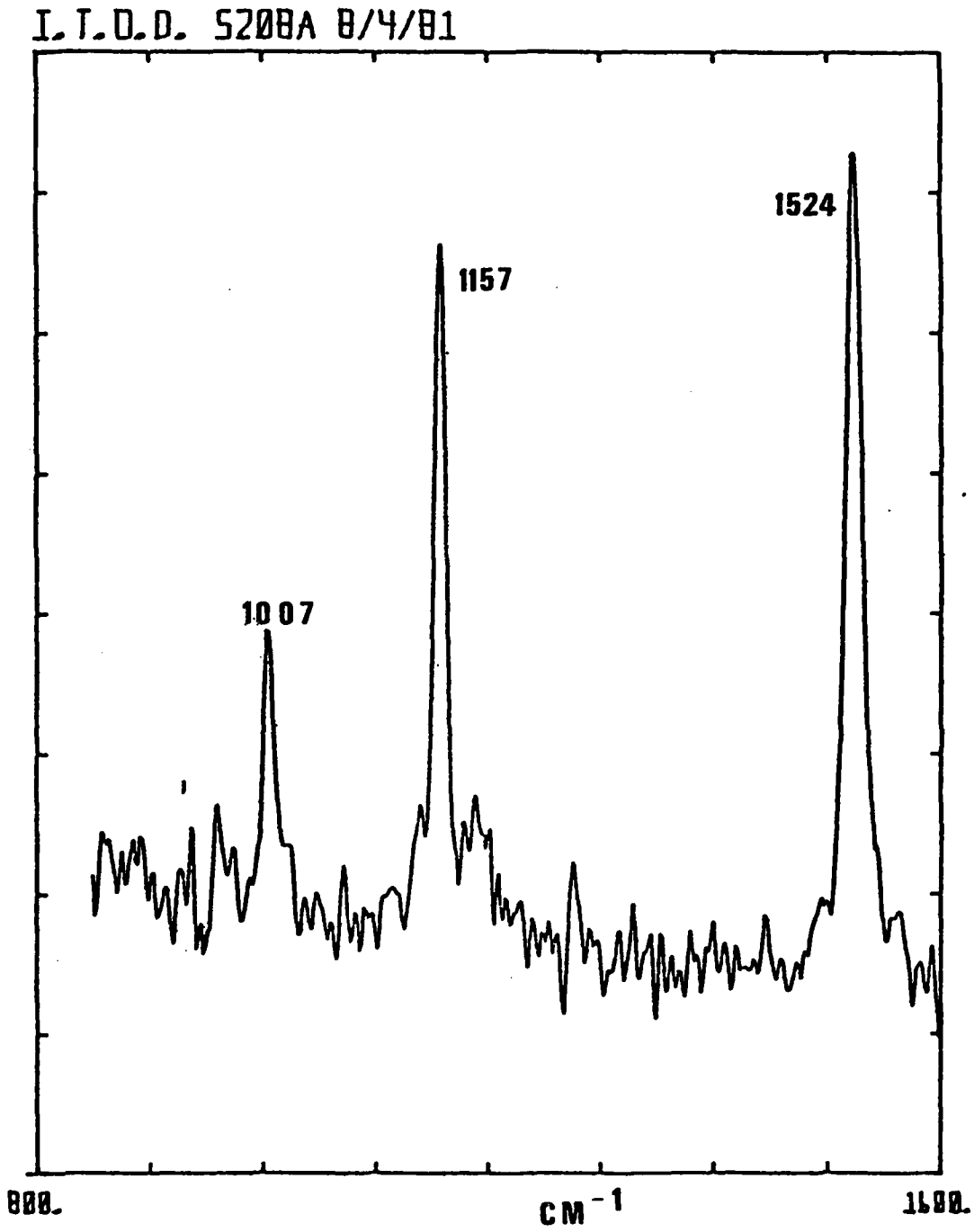

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Figure 1 The resonance Raman spectrum of intact turtle cone oil droplets with 520.8 nm excitation

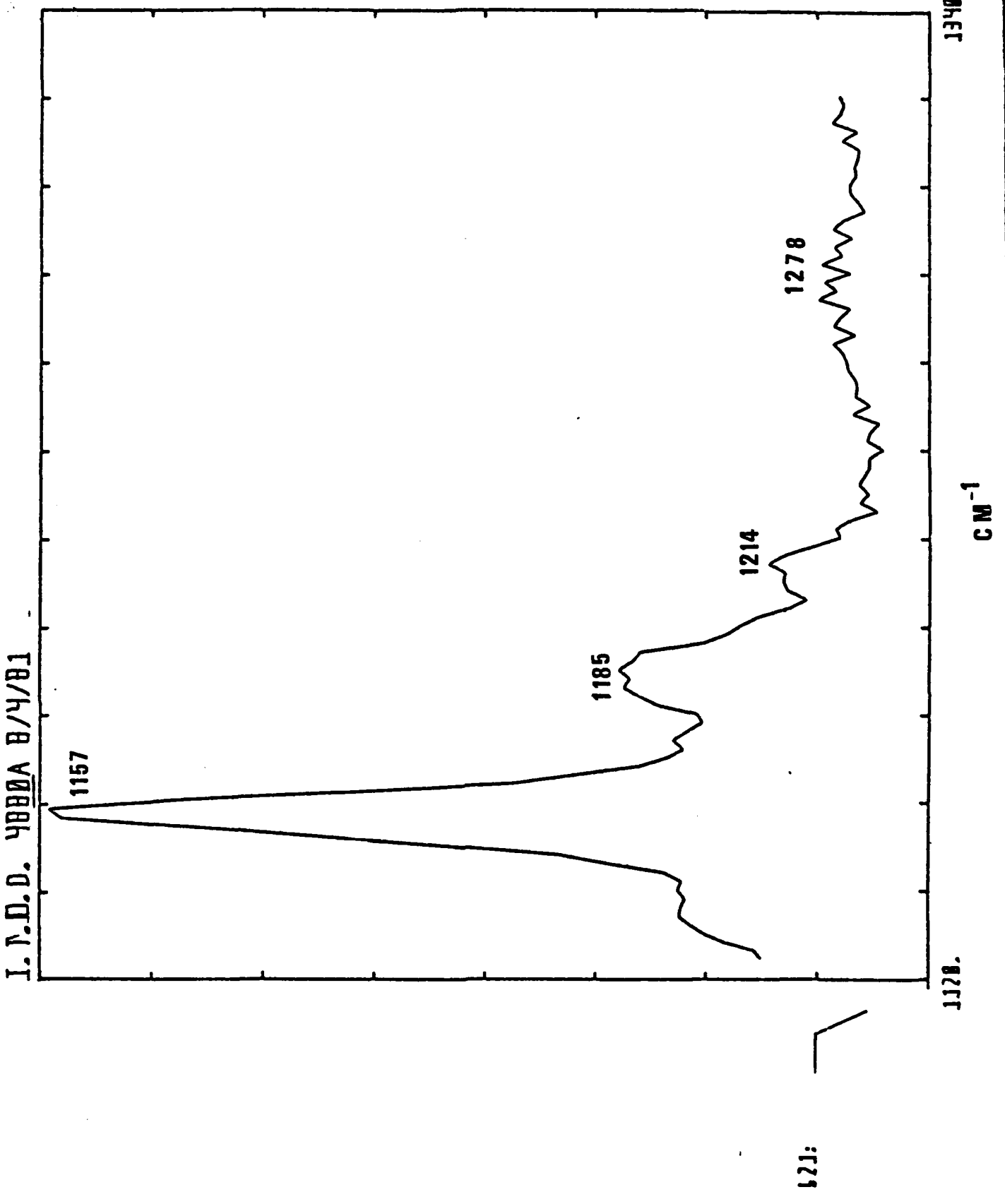

Figure 2 A high resolution cone oil droplet spectrum obtained with 488.0 nm in the limited wavelength region 1120 cm^{-1} to 1340 cm .

1278 cm^{-1}. This band only occurs if large amounts of the carotenoid astaxanthin are present.

The most important result of this study is seen in Figure 3. In this figure the intensities of the three prominent Raman lines are plotted as a function of different laser excitation frequencies. In other words at each laser excitation frequency the intensities of the three peaks were measured and then plotted as a function of these excitation frequencies. One thing is immediately obvious from these measurements -- the complete absence of a maximum in this plot at 520.8 nm. In fact if the oil droplets were composed of free carotenoids detailed theoretical studies have shown that such a graph should show a maximum at 520.0 nm (5). The reason for the lack of such a maximum can only be ascribed to the presence in the oil droplets of carotenoid excited state interactions which result, as has been shown in model systems by Lewis et al (4), from specific structural effects caused by either aggregation or the presence of specific carotenoid binding proteins.

A further proof of this hypothesis is alteration in the frequency of the carotenoid C=C stretching frequency with the wavelength of the exciting laser. This frequency is observed at 1524 cm^{-1} in Figure 1. Table I lists the position of this band for various excitation laser wavelengths along with the position of the other two major bands in the spectrum. Theory and model system experiments (4,5) show that for such carotenoids in aggregates a maximum C=C stretching frequency will be observed at 488.0 nm excitation and a minimum frequency will be detected at 568.2 nm. This is precisely what is observed in Table I and, as predicted (4,5) the other vibrational modes are unaffected by this effect.

Thus, so far, our studies have definitively shown that the carotenoids in the cone oil droplets are in large aggregates. Therefore, the anomalous blue absorption of the oil droplets where carotenoids have no absorption can now

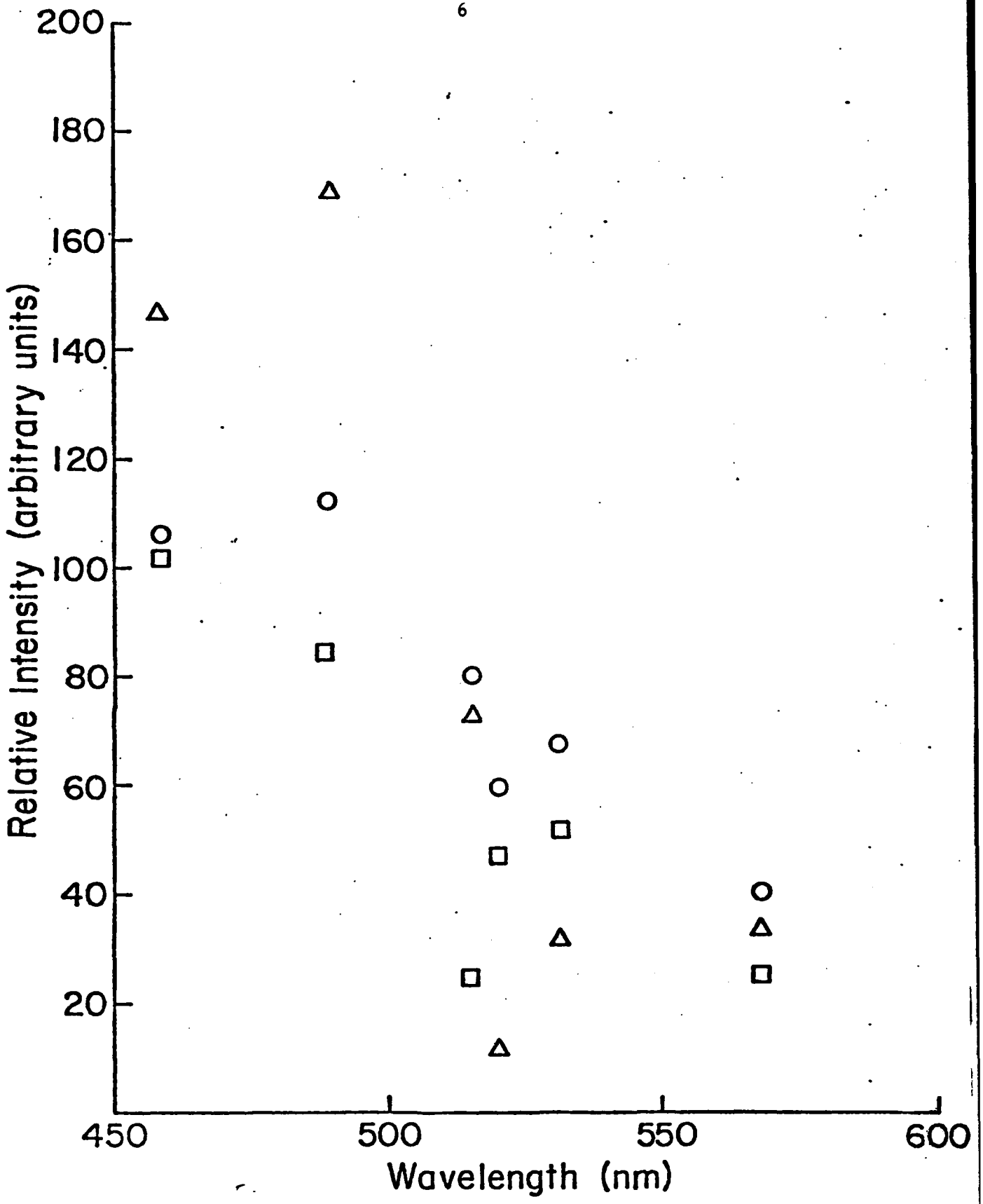

Figure 3 Excitation profile for the three main carotenoid vibrational modes in intact turtle oil droplets. Δ - 1515 cm^{-1}, O - 1158 cm^{-1}, □- 1006 cm^{-1}.

TABLE 1

Dependence of the three main Raman vibrational modes of intact turtle oil droplets on excitation frequency

λ	a	b	c
4579	1524	1004	1156
4880	1528	1005	1158
5145	1522	1004	1156
5208	1524	1007	1158
5309	1521	1007	1157
5682	1517	1005	1157

be understood as being caused by an excited state "exciton" interaction resulting from large aggregates. This exciting result is the first implication of exciton interactions in visual systems even though such interactions have been known to exist in all photosynthetic systems.

It has been suggested(6,7) that turtle cone oil droplets serve the same function as the macular pigment in primate retinas: the elimination of the out-of-focus blue image. The optical system in vertebrates is not achromatic. There is a difference between the focus point for red and blue light, which is approximately 1 diopter for the human eye (8). When an object is illuminated by white light, and the eye is focused for light in the yellow or red part of the spectrum, there will be an out of focus blue image present. The cone photopigments which are sensitive in the yellow and red have a secondary absorption peak in the blue, known as the cis peak (9), which would have amplified this effect. The macular luten region of the primate retina (the center of which is the forea, where visual activity is the highest) contains a yellow pigment, which is highly abosrbing in the blue, and serves to eliminate the out-of-focus blue image.

For efficient filtering, high abosrbance down to 4000 Å is needed. Carotenoid absorbance at this wavelength is usually less than 10% of peak absorbance, while exciton interactions in astaxanthin, zeaxanthin and lutein aggregates would readily shift the peak absorbance to near 3000 Å. Thus, the presence in cone oil droplets of various degrees of aggregation assures exciton interaction between the carotenoid molecules and therefore high absorbance over the whole blue portion of the spectrum.

Investigations are just beginning and proposed(below) for the coming year to study turtle retinas damaged under LAIR specifications and to investigate the role in damage mechanisms of this unique, high absorbing, effective

filtering oil droplet system through which all light entering the turtle's visual cells must pass.

II. A Selective Probe of Membrane Potentials in Turtle Cone Cells

The presence of carotenoids in the visual system of the turtle suggested to us another important avenue by which we could investigate laser damage mechanisms. To understand this additional aspect of our work on turtle retinas recall that the resonance Raman spectrum of carotenoids results from a $\pi\pi^*$ transition of the molecule when it encounters wavelengths of light close to its lowest energy electronic transition. Membrane potential changes have been shown to alter this electronic transition and these alterations are readily detected as perturbations of the resonance Raman spectrum(10). Thus the resonance Raman spectrum of carotenoids is sensitive to membrane potential and it occured to us that this spectrum of carotenoids in the turtle retina could be responsive to the membrane potentials in turtle visual cells. Of course these membrane potentials are intimately connected with the detailed molecular events that establish visual response and therefore should be very sensitive to laser induced molecular damage in the visual cells.

To test this hypothesis that the carotenoids we detected in the visual system of the turtle could pick out light induced membrane potential alterations we performed a series of kinetic experiments. In these experiments the resonance Raman spectrum of the carotenoids in whole turtle retina were detected kinetically at certain time intervals after rhodopsin light excitation. The data we obtained is seen in Figure 4. Only vibrational modes of carotene are observed (11,12). Vibrational modes of rhodopsin (13) are absent. Retinas exposed to a stimulating light 11 μs and 14 μs before the carotenoid spectrum was detected were found to have I_{1157}/I_{1520} ratios of 1.02 and 0.98, respectively;

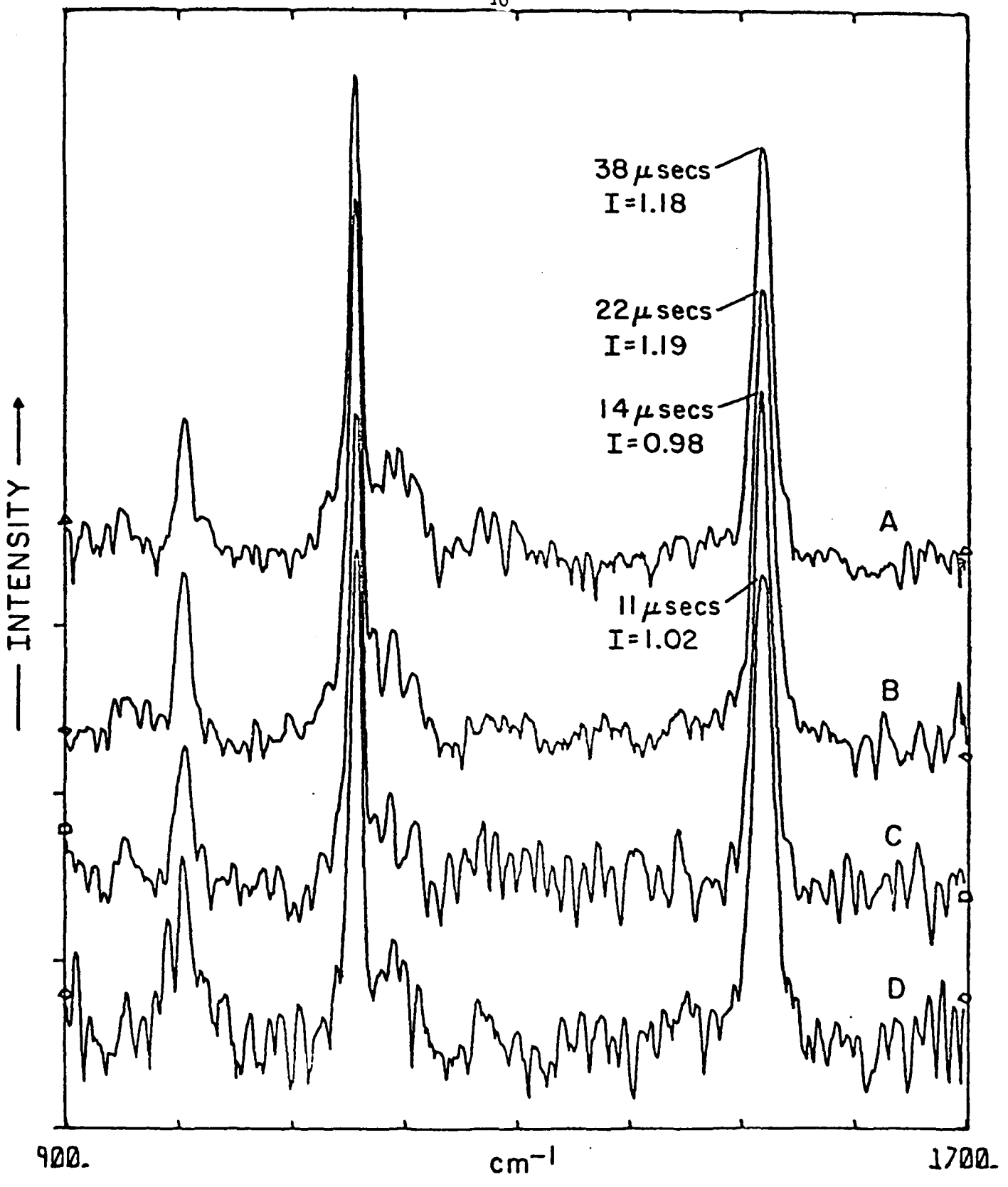

Figure 4 Kinetic resonance Raman spectroscopy of carotenoids in turtle retina with various time delays after a stimulating light pulse. Spectra were taken with 514.5 nm excitation and I = intensity ratio of the 1157 cm^{-1} band to the 1520 cm^{-1} band.

whereas the ratios were 1.19 and 1.18 for retina exposed 22 μs and 38 μs before the carotenoid spectra were obtained. Based on these data, it appears that the resonance Raman spectrum of carotene is sensing a rapid light-induced membrane potential in these retina.

To test this hypothesis, valinomycin and nigericin were added to the retina to prevent membrane potential formation and spectra were again recorded with a 38 μs delay after light excitation. Data comparing retina without and with valinomycin and nigericin at 38 μs time are seen in Figures 5A and B. It is clear from these spectra that valinomycin and nigericin addition to the retina influences the I_{1157}/I_{1520} ratio. Furthermore, when spectra were obtained at 38 μs and 11 μs delay times with retina containing nigericin and valinomycin, the change in the I_{1157}/I_{1520} ratio was abolished (Figures 5B and C). These data indicate that the changes seen in Figure 4 reflect the establishment of a rapid light-induced membrane potential.

In summary we have established that the resonance Raman spectrum of carotene is a sensitive monitor of kinetic membrane potential changes even in a highly scattering medium such as turtle retina. As we have demonstrated, the kinetic membrane potential changes caused alterations in the <u>intensity</u> of the 1157 cm^{-1} ($\doteq$C-C=) vibrational mode and the 1524 cm^{-1} (-C=C-) vibrational mode. No changes in the <u>frequency</u> of these vibrational modes were observed. It is a well accepted fact (14) that frequency alterations reflect ground state conformational changes whereas intensity alterations are characteristic of excited state perturbations. Our data clearly demonstrate that the carotene potential-sensitive spectral changes originate as a result of excited state, electrochromic perturbations. Thus, the data provide good evidence that our measurements were not limited by the time response of the carotenoids in the retina. Their response time should be <picoseconds (10^{-12}s) which is the approximate time scale of the resonance Raman scattering process.

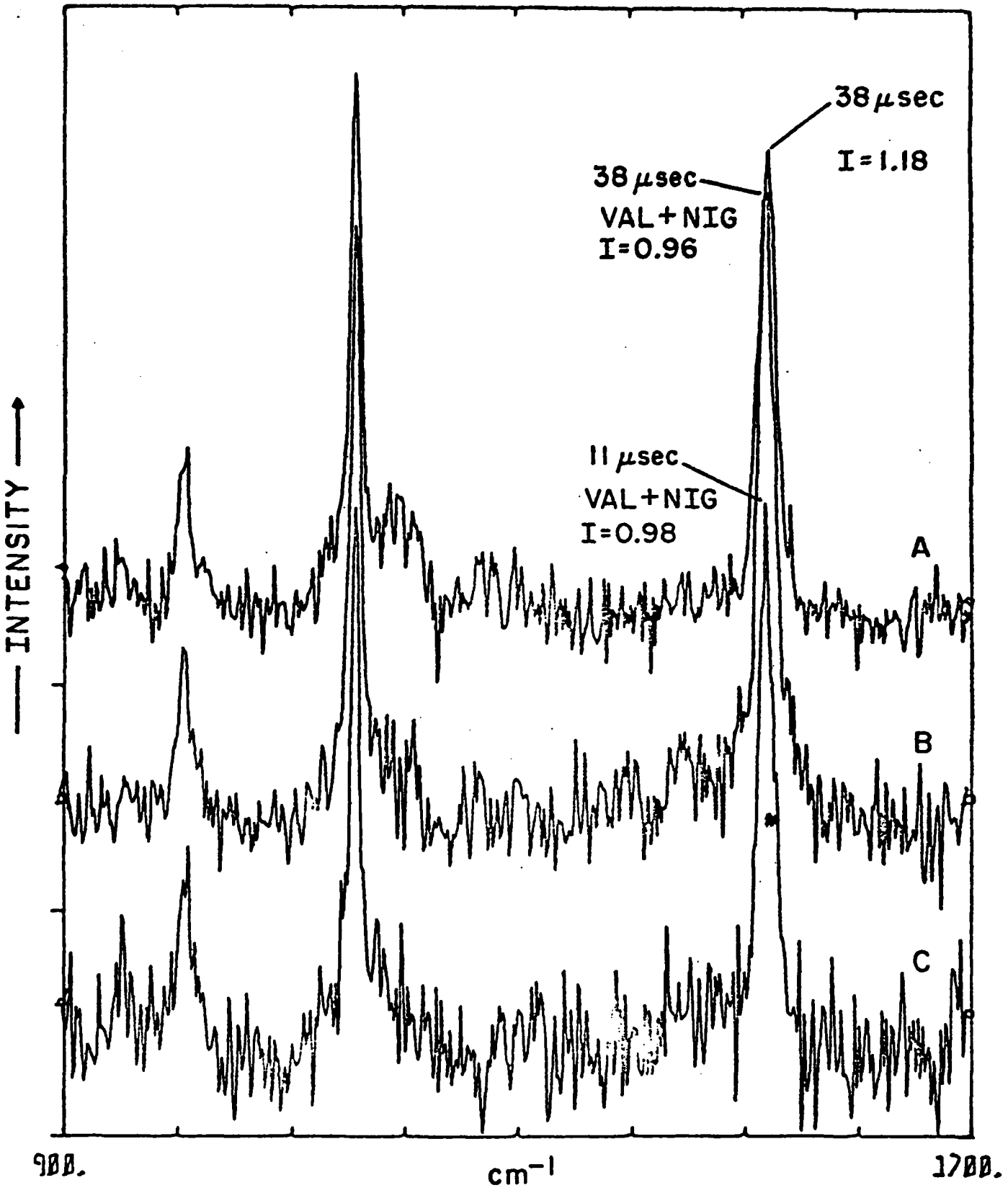

Figure 5 A comparison of the kinetic resonance Raman spectra of carotenoids in turtle retina obtained with a 38 μs delay after light excitation (A) without and (B) with valinomycin and nigericin and at 11 μs with valinomycin and nigericin (C). Spectra obtained with the same laser frequency and I has the same definition as in Figure 4.

This ability of the turtle retinal caretenoids to be a sensitive selective monitor of photoreceptor membrane potentials is an important extension of our oil drop studies and should be a sensitive probe of the most subtle biochemical alterations which result from low level laser radiation damage. These studies will be initiated in the next few months of the contract period and will continue into the next contract year.

III. Angstrom Resolution Light Microscopy of Photoreceptor Cells

Significant strides have been made in this aspect of our research during the past several months of the contract year. Firstly after approval of the contract in February a microscope was purchased and set up to our specifications. Since then we have been obtaining important preliminary information using labeled antibodies that we had produced earlier under this contract (see last year's contract).

Figure 6 is a photograph of a fluorescently labeled rod outer segment (ROS) from bovine retina. These ROS are labeled with rhodamine labeled antibodies against rhodopsin. The antibodies were isolated using the following procedure: Pure bovine rhodopsin was suspended in 0.2 M phosphate buffer pH 7.0 and used as the immunizing agent. This solution containing approximately 1.0 mg bovine rhodopsin was homogenized in an equal volume of Freund's complete adjuvant and then injected into the footpads of each rabbit. After three weeks these rabbits received first an intraperitoneal injection of 1.2 mg rhodopsin solution and two days later the same amount of antigen injected intravenously. The rabbits were bled twice on the 5th and 10th day after the intravenous injection and antisera were obtained. The antibodies were purified by published methods (15). The antibodies were then labeled with a rhodamine label and introduced into the ROS by fusing vesicles of phosphotidylcholine containing the

Figure 6 Rod outer segments labelled with rhodamine labelled antibodies against rhodopsin.

antibodies with ROS and then photographed with the Leitz fluorescent microscope.

Using similar methods two other critical components of bovine photoreceptor cells have been labeled. These are the light-activated phosphodiesterase and GTPase that have been discovered in rod outer segments (16). Bovine material was used for these preliminary studies because of its availability in sufficient quantities for detailed biochemical analysis. This biochemical analysis is an important part of our studies in order to understand fully the complexities of our labeling procedure. Detailed biochemical separations have definitely shown that the antibodies are labeling the specific cellular components they were designed for.

We have now viewed the fluorescence from these cells through specially designed submicron apertures. These apertures had 2000 Å, 1500 Å, 1000 Å and 800 Å dimensions and the results we have obtained have already begun to give us the baseline data that we can use to analyze alterations in photoreceptor molecular cytology altered by low level laser radiation. The data obtained by us is outlined in Table II. In this table we see that, in the dark, even with a 1000 Å aperture the fluorescence from all three species rhodopsin, GTPase and phosphodiesterase can be seen whereas after the cells were illuminated with 10^{11} quanta/cm^2/sec at 600.0 nm only with the 1500 Å aperture can all three species be detected. However, the addition of HEPES buffer to the cells and using the same intensity light as was used in column 2 of Table II all three species could be observed again even with the 1000 Å aperture. If, on the other hand, 10 times the 10^{11} quanta/cm^2/sec light intensity was used even in the HEPES treated cells once again similar results were obtained as with the untreated photoreceptors(column 1). Cells with their pH raised to 10.2 give similar results as the HEPES treated cells. No alterations of any kind could

TABLE II

Alterations in the Molecular Cytology of Photoreceptor Cells with Light and Chemical Agents

Aperture Size	D	L	L(HEPES)	10 x L(HEPES)	L (pH=10.2)	10xL (pH 10.)
2000 Å	RGP	RGP	RGP	RGP	RGP	RGP
1500 Å	RGP	RGP	RGP	RGP	RGP	RGP
1000 Å	RGP	RG	RGP	RG	RGP	RG
800 Å	RG	RG	RG	RG		RG

LEGEND: R, G and P represent, respectively, the presence of fluorescence from rhodamine labelled rhodopsin, GTPase and phosphodiesterase. D represents cells with no previous exposure to light and L represents cells which have been exposed to 10^{11} quanta/cm^2/sec at 600.0 nm.

be observed in these cells with an electron microscope study of the commonly observed cytological features.

The above results therefore demonstrate that even what has been considered completely safe laser illumination can cause discrete cytological molecular alterations of the photoreceptors. These cytological alterations are completely undetectable by electron microscopy and thus, highlight the powerful new role that angstrom resolution light microscopy will have in the elucidation of damage mechanisms and as a diagnostic tool to set important new definitions of laser standards. In addition, our studies are indicating that certain simple chemical alterations in the cellular chemistry (such as incubation with HEPES buffer or pH alteration) can prevent these laser induced cytological perturbations.

IV. Identification and Selective Staining of Other Important Molecular Components of Photoreceptor Cells.

A central question in our minds as we have approached the molecular aspects of light induced damage of photoreceptor cells was whether the structural protein actin could be involved. However, there have been no reports indicating the presence of actin in photoreceptor outer segments. We therefore set about trying to determine whether actin could be present in photoreceptor outer segments. In order to determine this we used the recently discovered mushroom phallotoxin phallacidin which strongly binds to F-actin and G-actin oligomers ($K_d \sim 2 \times 10^{-8}$ M) (15). This toxin internalizes into cells without any alteration required in plasma membrane permeability (15). The phallacidin has been made fluorescent by chemically coupling nitrobenzoxadiazole (NBD) (15) and the combination NBD-phallacidin has been added to photoreceptor cells. After incubation staining is achieved as seen in Figure 7. In this figure two outer segments are seen. These are outer segments obtained from frog (Rana catesbiana) retina. Frog retinas were chosen because we wanted large, readily available photoreceptors for these preliminary experiments before moving into species of

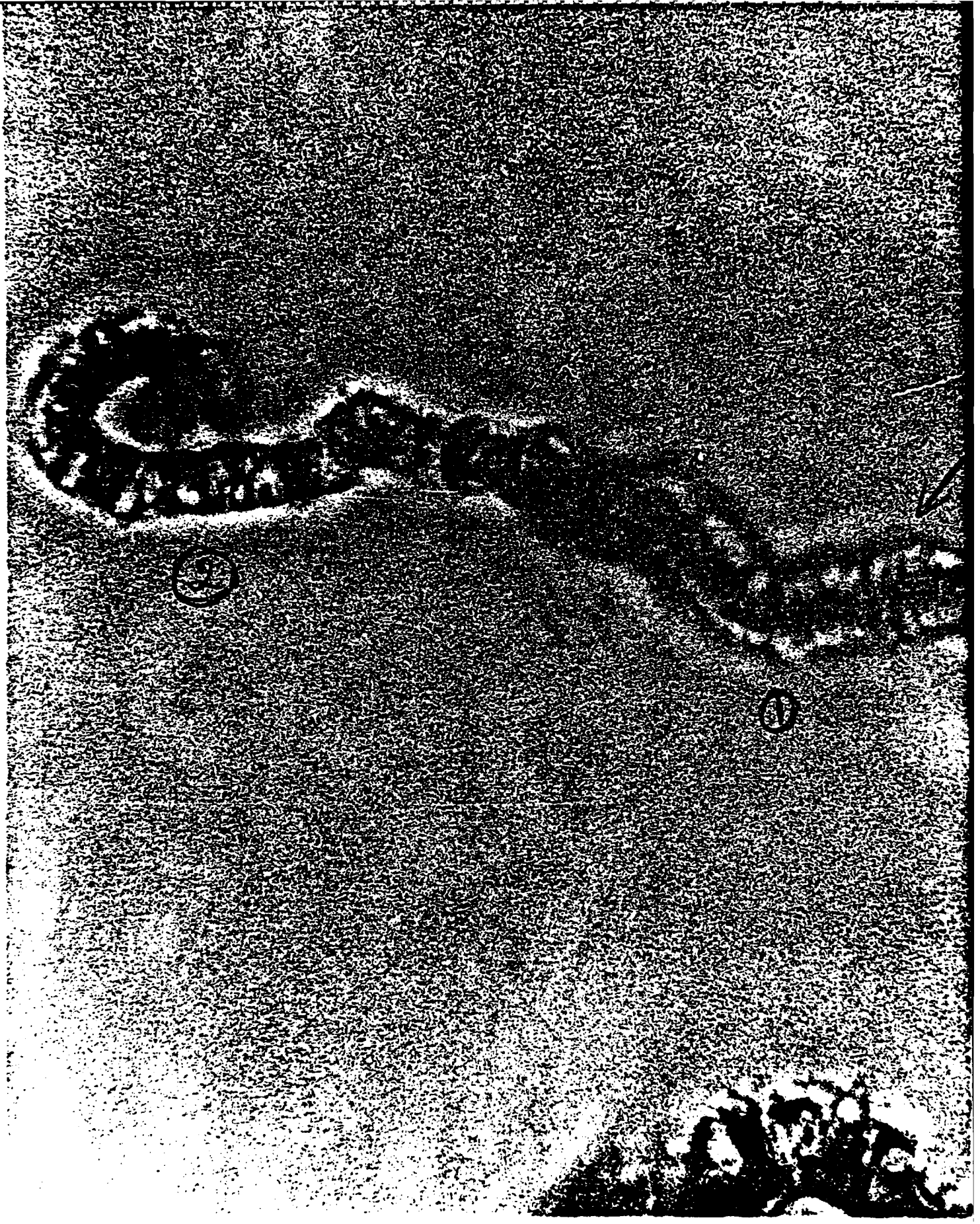

Figure 7 Two photoreceptor outer segments from Rana catesbiana indicated by (1) and (2) on the picture and labelled with the actin specific dye NBD-phallacidin.

interest to the Army. An arrow marks one of the outer segments where definite staining is observed around the discs of the outer segments. A control experiment in which first non-fluorescent phallacidin is added eliminates the staining by NBD-phallacidin. In addition each outer segment is surrounded by a halo of fluorescence from dye which is not internalized and surrounds the plasma membrane of the cell.

Thus, during the past several months, in addition to rhodopsin, GTPase and phosphodiesterase our molecular cytology experiments have also lead to the important conclusion that actin is also present around disc membranes in photoreceptor outer segments. Therefore we now have selective staining procedures for 4 important components in photoreceptor outer segments and we will thus be able to apply angstrom resolution light microscopy to each of these components in our studies of laser induced damaged retinas in the next contract year.

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