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A FUNCTIONAL APPROACH TO THE ASSESSMENT OF OCULAR HAZARDS OF LASERS

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INTRODUCTION

The use of low energy lasers in research, industry, medicine and the military has increased dramatically in the past decade. As a consequence, the danger of laser-induced ocular dysfunction due to both thermal and nonthermal (photochemical) mechanisms has likewise increased (Ham et al., 1979). Current safety guidelines for laser exposures have been determined primarily by histopathological techniques and subsequent thermal burn calculations. While this approach is entirely appropriate for coherent quantal fluxes resulting in thermophysical lesions ($\approx 10^{21}$ to $10^{22}$ quanta/cm$^2$/sec.), these techniques are too insensitive to detect non-thermal effects on retinal function. The damage mechanisms involved in non-thermal effects probably involve photoactivation of molecules leading to membrane oxidation.

A more sensitive approach, electrophysiological recordings of the electroretinogram (ERG), has been used to monitor the functional viability of the retina, per se. This bioelectric response to light is a massed voltage potential recorded from the entire eye. Since the ERG correlates well with overt visual function, it has proven to be a useful tool in both clinical and research settings. However, since it represents neural information summed over the

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entire retinal surface, it cannot be used to differentiate between contributions
of separate retinal loci. Therefore, any localized retinal dysfunction will
usually go undetected (except in the macula). Since laser irradiation is
focused onto the retina in the same manner as incoherent light, the spatial
dimensions of the irradiated tissue can remain relatively small and, therefore,
escape detection by usual ERG analysis methods.

We have undertaken the task of quantitatively reassessing current laser
exposure safety standards using sensitive neurophysiological techniques which
can selectively probe regional differences in functional behavior across
the retina. We will assess any acute non-thermal laser effects in an in
vitro retinal preparation that allows rigorous control over many important
optical and physiological variables while retaining its visual responsivity
for hours. This report details the techniques involved and the methods of
our data endpoint analysis. The following paper (Kong and Raybourn, in
preparation) will present our findings with the HeNe laser.

METHODOLOGY

Our approach involves the quantitative analysis of Intensity (stimulus)-
Response (biological), or I-R, functions obtained with electrophysiological
recordings from neural tissues in an in vitro "retinal eyecup" preparation.
We use two independent recording microelectrodes (in conjunction with coaxial
incoherent and laser light stimulation) to monitor two different retinal
sites. This provides an all-important intra-retinal control hitherto unobtainable
in risk assessment studies. In this way we can determine the dose-response functions for safe levels of incident electromagnetic energies using functional endpoints instead of the more classical, but less sensitive, histopathological techniques. This approach has also been adopted to study acute effects of magnetic field exposures in a related DOE-funded project (Raybourn, 1981).

**In Vitro Preparation**

For our model system, we are using the reptilian retina of the turtle. The rationale for this choice is three-fold. First, all vertebrate retinas are basically identical in the way(s) in which they transduce incident light into meaningful neural signals. Secondly, save for the erythrocyte, more is known about the biophysics and biochemistry of the turtle photoreceptors than any other vertebrate cell. Lastly, the physical dimensions of the turtle photoreceptors are large enough (5-8 μm x 75 μm versus 0.8 μm x 80 μm in man) to enable eventual intracellular recordings for direct assessment of membrane functions.

In order to assess differences in susceptibility of photoreceptor types (rods, cones), two species of turtle are being used: The red-eared, or common swamp turtle, *Pseudemys scripta elegans*; and the snapping turtle, *Chelydra serpentina*. The former has a predominantly cone photoreceptor-type retina (over 95%), while the latter has a mixed rod-cone photoreceptor-type retina (40% and 60%, respectively).
Our surgical procedure is as follows: At a specific time in an artificially maintained 12 hour light: 12 hour dark diurnal cycle, the animal is decapitated and pithed. Under dim red light, the eye is dissected from the head and hemi-sected in a plane perpendicular to the anterior-posterior axis, near the ora serrata. The anterior portion of the eye is discarded while the posterior portion is drained of its vitreous humor, leaving the so-called "retinal eyecup". This in vitro preparation can retain its physiological viability for up to 5 hours, although 3-4 hours is our usual experimental duration.

Recording Techniques

The eyecup is placed in a lucite recording chamber into which a water-saturated mixture of \( O_2 : CO_2 \ (95\%:5\%) \) gas is delivered. The scleral (back) portion of the eyecup is grounded via Ag-AgCl contacts in the recording chamber. Glass microelectrodes used for recording ERG's are made on a Livingston-type pipette puller, filled with .2m NaCl, and have resistances of about 250 Kohms. Two recording electrodes are positioned onto the retina via independent hydraulic drives. Signals from each electrode are amplified via separate recording channels and conventional a.c. recording techniques are used. Data is stored on magnetic tape and on computer diskette for future playback and analysis (see below).

Optical System

Incoherent white light is provided by a 200 W tungsten-iodine lamp (1.8 \( x 10^{14} \) quanta/sec/cm\(^2\) at 635 nm) in a two-channel optical system. Intensity and wavelength are controlled by interposing neutral density and narrow-band
interference filters (20 nm half-width). Two apertures in the optical pathway, each with 2-dimensional mobility, are used to create two separate focussed images on the retina (Figure 1). Thus, a separate stimulus spot can be centered on each recording electrode. The two spots are individually shown such that the stimuli can be alternately presented. Stimulus timing is controlled by a digital pulse timer which triggers Uniblitz drivers and electromagnetic shutters.

Several laser devices are being used to provide laser stimulation, including a 0.5 mW and a 2 mW Helium-neon (HeNe) laser, and a 0.6 mW Gallium Arsenide (GaAs) semiconductor diode laser. With all lasers, the laser beam is combined with the incoherent light path by means of a beam splitter. Thus, coherent and incoherent beams are coaxial. Positive and negative lenses are used to match coherent and incoherent retinal image sizes. Since the laser exposure durations in our experiments are on the order of several minutes, manual shuttering of lasers has proven adequate.

Description of ERG

The electroretinogram (ERG) is a field potential measured across the retina in response to light. The ERG is composed of several components, each generated by a different retinal cell type (or types). The components of interest in our studies will be the early receptor potential (ERP), and the a-wave and b-wave of the ERG.
Figure 1 - Photograph of the retinal eyecup preparation showing separate test and control light spots with respective recording electrodes. Each spot is 1 mm in diameter. Photographed through a dissection microscope at x10 magnification.
The ERP is a very short latency response (less than 25μ sec.), consisting of two components, R₁ and R₂. R₁ is a positive (with respect to cornea) wave, while R₂ is negative. The ERP is derived from the initial charge displacements of the photopigment in the photoreceptors due to photon absorption. The ERP amplitude is linearly proportional to the amount of unbleached photopigment. The ERP is resistant to anoxia and alterations of the ionic environment. The nature of the ERP generation (charge displacements in photopigments) makes it an invaluable probe for studying the interaction of incident electromagnetic irradiations with the biophysical mechanisms of visual transduction at the level of the photosensitive molecules.

The a-wave (or P-III) is derived from the response of the entire photoreceptor cell to light and is dependent on non-linear, ionic conduction mechanisms (Penn and Hagins, 1972). In contrast to the ERP, the a-wave is quite sensitive to metabolic inhibitors and alterations in the ionic environment. The polarity of the a-wave (corneal negative) is the same as the R₂ component of the ERP and often masks the ERP, unless special experimental protocols are employed.

The b-wave of the ERG is the most widely used electrophysiological measure of retinal function and viability. It is a large positive wave with a latency longer than the a-wave and consequently masks much of the a-wave. The exact source of the b-wave is not known, but it is generally believed to reflect neural activity proximal to the photoreceptors.
(bipolar and horizontal cells, primarily). Some evidence exists which suggests glial (Muller) cell sensitivity to external potassium concentration changes is involved (Miller and Dowling, 1970).

Computer Analysis

Electroretinograms are stored in digital form by means of a Cromemco analog-to-digital converter and Mountain Hardware real-time clock interfaced with a Northstar Horizon 2 microcomputer. The sampling frequency is 1KHZ. The computer also performs such data processing functions as data extraction and conversion into I-R function format, calculation of spectral sensitivity values, and graphic display of I-R and spectral sensitivity plots via an Epson MX-70 dot-matrix printer with graphics capability.

ADVANTAGES OF PREPARATION

1. **Intra-retinal Control.** The ability to use two separate recording electrodes with two separate stimulus spots allows us to monitor the local functional condition of two different retinal sites simultaneously. This provides a very important intra-retinal control. When one retinal site is exposed to laser irradiation, we can compare the response properties of the irradiated (test) site before, and after laser exposure with that of the non-irradiated (control) site. This enables us to determine whether response changes at the test site are due to the laser irradiation or to other general factors affecting the entire retina such as deterioration of the preparation. Previous laser studies relied upon comparisons
between different retinae, or animals, when assessing retinal function.

2. **Specification of Stimulus Parameters.** The use of a retinal eyecup preparation greatly facilitates the specification of light stimulus parameters at the retinal plane. Stimulus size is easily determined using pre-calibrated microscope graticules. Stimulus intensity measurement is simple because factors such as the transmission and scatter properties of usually intervening ocular components (cornea, lens, vitreous, etc.), which are important considerations in the in vivo preparation, can be ignored. Retinal location is also easily determined by microscopic examination of the eyecup (see Figure 1).

3. **Pharmacological "Dissection" of the ERG.** The ERG is a waveform which is composed of different components that can be individually dissected out by the use of various pharmacological agents. For example, aspartate, GABA, cobalt, and glutamate are effective in eliminating the b-wave component of the ERG, leaving the unobscured a-wave reflecting "pure" photoreceptor activity (Sillman, Ito, and Tomita, 1969). In the eyecup preparation, chemicals such as aspartate or Co^{++} can be easily administered, either by perfusing, atomizing, or pre-incubating the eyecup in the desired medium. Figure 2 shows the ERG derived from a normal, untreated eyecup and an ERG obtained from an eyecup which was preincubated for 15 minutes in 30 mM aspartate ringers. (In pre-incubation, the chemical is placed in the eyecup for several minutes and then removed prior to recording.) The b-wave is completely blocked by aspartate, leaving
Figure 2 - ERG's recorded from both a normal (upper) and an aspartate-treated (lower) eyecup. The aspartate ERG exhibits a prominent a-wave (negative polarity) but no b-wave. Stimulus onset is indicated by an upward deflection of the bottom trace.
only the negative polarity, photoreceptor-generated a-wave. This technique of pharmacological isolation provides an important method for differentiating photoreceptor effects from synaptically-mediated retinal effects.

4. **I-R Analysis of Entire Physiological Response Range.** Changes in retinal sensitivity over the entire dynamic response range of the retina are quantified using the I-R function. Earlier studies sampled only a single point along this range (see below). The I-R function analysis was adapted from systems engineering concepts utilized in electrical circuit design and analysis (Werblin and Dowling, 1969).

5. **Intracellular Recordings.** A further advantage of the eyecup preparation is its adaptability to intracellular recording techniques. The use of intracellular probes in conjunction with current-injecting circuitry provides a potentially powerful tool for assessing mechanisms of laser effects at the membrane level. This is an all-important capacity if non-thermal (photochemical) laser effects involve membrane function. We are currently in the process of incorporating this capability into our system.

**DATA ANALYSIS**

Our method of assessing retinal function is based on the analysis of the photic stimulus intensity--biological response amplitude relationships...
of the locally-recorded ERG. Our data is presented in the form of intensity-response (I-R) functions. There are three major advantages in using I-R function analysis over the more familiar methods of tracking threshold criterion values. First, it measures retinal behavior over the entire physiological response range, not just at one point (e.g. threshold). Secondly, this analysis offers a rigorous method for defining and quantifying changes in visual sensitivity at different retinal sites. Lastly, I-R function analysis can reveal information about the cellular and/or synaptic mechanisms affected.

The I-R function is derived in our experiments by eliciting ERG's to brief (100 msec.) flashes of incoherent white, or chromatic, light of varying intensities. Figure 3 illustrates such an experiment. The I-R function is a plot of the amplitude of the ERG component of interest (R), which in this case is the b-wave, as the ordinate versus the log intensity of the test flash which elicited that ERG along the abscissa. This plot is shown by the filled circles in Figure 4. This plot follows a characteristic sigmoidal shape with the response first growing slowly with increasing intensities of light, increasing rapidly above -3.0 log units, and finally beginning to saturate above -1.0 log units. The steep portion of this curve represents that region of the retinal response range that maximizes biological signal: noise ratios (i.e. small intensity increments yield large responses). We can also characterize any retinal locus by its dynamic response range (in this case, the smallest response detectable above the noise level was 20 µV and the maximum response was 180 µV), and the intensity domain over which this dynamic response range occurred (3.5 log units in this experiment, from -4.0 log units to -0.5 log units).
Figure 3 - ERG's elicited by light flashes of varying intensities. The log relative intensity is indicated to the left of each response. Response traces have been displaced vertically for clarity.
Figure 4 - The I-R function derived from the experiment shown in Figure 3. The peak b-wave values are shown by the filled circles. The dimensions of the ordinate labelled "R" are microvolts. The hyperbolic tangent function (see text) is shown by the solid line.
This I-R function can be described more formally by the function:

\[ \frac{R}{R_{\text{max}}} = \frac{I^N}{I^N + \sigma N} \]

Where I is the test flash intensity, R is the response elicited at that intensity, \( R_{\text{max}} \) is the maximum response elicited (the saturated response amplitude), and \( \sigma \) is the intensity which elicited a half-maximal response, and \( N \) is a constant. The curve described by this expression is shown as the solid line in Figure 4, where \( N \) has the value of 0.8. The intensity response functions of all retinal neuronal types, determined by both intracellular and extracellular recording techniques, have been shown to be of this form, but with different values of \( N \) (Thibos and Werblin, 1978). The I-R function thus provides a detailed, quantitative characterization of the intensity-response properties of the retinal site studied. This is an important index in assessing changes in retinal function.

The I-R function also can be used to quantify changes in sensitivity. The I-R function in Figure 4, for example, represents the dynamic response range of a given retinal site, but this is true only at one particular level of ambient illumination. The intensity domain over which the retinal site responds can be extended by both cellular and synaptic adaptation mechanisms in response to changes in background ambient illumination. For example, in Figure 5, curve A shows the I-R function measured at a retinal locus in
Figure 5 - I-R functions obtained at two different ambient light levels. Curve A was measured in the dark (no background light). Curve B was measured in the presence of a -2.0 log unit background light. The half-maximal intensities for curves A and B, labelled $\sigma_A$ and $\sigma_B$, respectively, show a shift in sensitivity of approximately 1.0 log units along the abscissa.
the dark (no ambient light). When the I-R function was measured at the same
site in the presence of a background light of -2.0 log units, curve B was
obtained. This curve is shifted to the right along the intensity axis with
respect to curve A. This means that brighter test flashes are required to
elicit responses comparable to those obtained in the absence of the background
light. In other words, the sensitivity of the retinal locus has been reduced
due to the change in ambient light level. This process is referred to as
light adaptation. The change in sensitivity due to the background light
can be expressed quantitatively by taking the difference between the half-
maximal intensities, \( \sigma \), of the two I-R functions. In this case \( \sigma_A \) is -3.8
log units and \( \sigma_B \) is -2.8 log units, so there is a change in sensitivity of
1.0 log units.

Conventionally, sensitivity has been determined by defining a single
arbitrary response amplitude as the threshold criterion level, and measuring
the test intensity required to attain such a criterion response. Sensitivity
is then simply the inverse of the test intensity measured (by definition).
There are potential problems with this method. Figure 6 shows two I-R functions,
labelled 1 and 2. If a threshold criterion level of X is chosen, the threshold
intensities for curves 1 and 2 are A and B, respectively. Thus, by conventional
methods of sensitivity analysis, there is a difference in sensitivity between
curves 1 and 2. However, it is clear that the domain over which the tested
areas respond are identical (between -4.0 log units and 0.0 log units).
How can this discrepancy be resolved? If we take the half-maximal intensities
of the two curves, Y and Z for curves 1 and 2 respectively, we find both
Figure 6 - Schematic illustrating I-R function analysis as compared with single threshold criterion analysis (see text for description).
curves have the same $\sigma$ value and can conclude that there is, in fact, no difference in sensitivity between the two curves. This illustrates a basic problem with single criterion level threshold analysis: "sensitivity" changes often are illusory or mask other effects. For this reason, we prefer the I-R function method of sensitivity determination, wherein sensitivity is defined by the position of the I-R function along the intensity axis as represented by the half-maximal intensity.

IR function analysis can also provide information beyond the determination of sensitivity changes by shedding light upon the cellular mechanisms involved. For example, a compression of the I-R function (decrease in $R_{\text{max}}$ without a change in $\sigma$), as shown in Figure 6, suggests a saturation, or shunting, phenomenon somewhere in the chain of events producing the response (e.g. shunting of the membrane due to an increase in membrane conductance).

SUMMARY

This report describes electrophysiological techniques that have been developed in order to re-assess ocular exposure safety standards for low energy laser (and, eventually, low dose ionizing radiation) exposures. Since low energy laser effects on ocular tissues are not based on thermal damage considerations, the more classical histopathological techniques are inadequate to realistically assess these non-thermal (photochemical) effects. The approach used in our research monitors the functional status of retinal tissues and, thus, provides a quantitative analysis of dose response relationships without relying on cell death or thermal burn criteria.
The use of this in vitro retinal eyecup preparation allows precise optical dosimetry at the retinal plane. Also, the use of two independent recording electrodes within the same eyecup allows an intra-retinal control which uniquely compares any local effects in irradiated regions against a non-irradiated control site. In this way, the direct dose-response relationships between laser (or ionizing radiation) exposures and the functional capacity of retinal tissue can be determined.

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